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**Nutrition for post-exercise recovery and training adaptation**

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# **NUTRITION FOR POST-EXERCISE RECOVERY AND TRAINING ADAPTATION**

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department for Health

March 2016

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## ABSTRACT

The importance of post-exercise recovery nutrition has been well described in recent years leading to its incorporation as an integral part of training regimes in both athletes and active individuals. Muscle glycogen depletion during an initial prolonged exercise bout is a main factor in the onset of fatigue and thus the replenishment of glycogen stores is central for post-exercise recovery. Nevertheless, nutritional recommendations, particularly related to the precise nutrient amount/type to optimise short-term (2-6 h) recovery remain incompletely elucidated. Furthermore, the available nutritional guidelines to maximise muscle glycogen availability within limited recovery are provided under the assumption that similar fatigue mechanisms (i.e. muscle glycogen depletion) are involved during a repeated exercise bout, yet this has never been established. One strategy with the potential to accelerate muscle glycogen resynthesis and/or functional capacity is the co-ingestion of protein with carbohydrate. Notwithstanding this, there is outstanding need to establish the influence of carbohydrate-protein ingestion on recovery from running exercise. Beyond the acute post-exercise period, protein ingestion following exercise is known to increase muscle protein synthesis and thus modulate exercise-induced training adaptation. However, whether post-exercise protein ingestion increases the magnitude of running-based endurance training adaptation has not been determined. The collection of work presented in this thesis provides evidence that the availability of muscle glycogen at the end of recovery determines the capacity for repeated exercise such that muscle glycogen depletion is shown to be a major determinant of fatigue during this bout. Moreover, the present work suggests that energy intake *per se* and not macronutrient composition (i.e. the addition of protein) determines maximal muscle glycogen resynthesis rate and the capacity for repeated exercise. The final experimental chapter in this thesis also provides some evidence for the potential role of post-exercise protein ingestion in amplifying cardiovascular training adaptation through an increase in plasma albumin content in response to six weeks of prescribed endurance training when compared to an energy-matched carbohydrate supplement.

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# TABLE OF CONTENTS

<b>CHAPTER 1 .....</b>	<b>17</b>
<b>INTRODUCTION .....</b>	<b>17</b>
<b>CHAPTER 2 .....</b>	<b>21</b>
<b>REVIEW OF LITERATURE .....</b>	<b>21</b>
<b>2.1 SUBSTRATE METABOLISM DURING PROLONGED ENDURANCE-TYPE EXERCISE.....</b>	<b>21</b>
2.1.1 MUSCLE GLYCOGEN METABOLISM DURING PROLONGED EXERCISE .....	21
2.1.2 EXTRA-MUSCULAR CARBOHYDRATE METABOLISM DURING PROLONGED EXERCISE .....	26
2.1.3 LIPID METABOLISM DURING PROLONGED EXERCISE .....	31
2.1.4 PROTEIN METABOLISM DURING PROLONGED EXERCISE.....	34
<b>2.2 MECHANISMS OF FATIGUE DURING PROLONGED ENDURANCE-TYPE EXERCISE.....</b>	<b>36</b>
2.2.1 MUSCLE GLYCOGEN DEPLETION DURING PROLONGED EXERCISE .....	36
2.2.2 HEPATIC GLYCOGEN DEPLETION AND HYPOGLYCAEMIA DURING PROLONGED EXERCISE .....	39
2.2.3 DEHYDRATION AND HYPERTHERMIA DURING PROLONGED EXERCISE .....	40
2.2.4 CENTRAL MECHANISMS OF FATIGUE DURING PROLONGED EXERCISE .....	42
<b>2.3 POST-EXERCISE RECOVERY .....</b>	<b>46</b>
2.3.1 GLUCOSE TRANSPORT .....	46
2.3.1.1 Contraction-induced glucose uptake.....	46
2.3.1.2 Insulin-induced glucose uptake .....	49
2.3.2 SKELETAL MUSCLE GLYCOGEN .....	52
2.3.2.1 Glycogen structure and localisation.....	52
2.3.2.2 Glycogen resynthesis .....	54
2.3.2.3 The two phases of muscle glycogen resynthesis.....	56
<b>2.4 NUTRIENT INTAKE AND MUSCLE GLYCOGEN RESYNTHESIS .....</b>	<b>57</b>
2.4.1 AMOUNT OF CARBOHYDRATE INTAKE .....	58
2.4.2 TYPE OF CARBOHYDRATE INTAKE.....	59
2.4.3 TIMING OF CARBOHYDRATE INTAKE .....	61
2.4.4 PROTEIN CO-INGESTION WITH CARBOHYDRATE.....	63
2.4.4.1 Protein co-ingestion and insulin secretion .....	63

2.4.4.2 Protein co-ingestion and glycogen resynthesis .....	65
<b>2.5 RESTORATION OF EXERCISE CAPACITY FOLLOWING SHORT-TERM RECOVERY .....</b>	<b>70</b>
2.5.1 EFFECT OF CARBOHYDRATE INGESTION ON TIME TO EXHAUSTION .....	70
2.5.2 EFFECT OF PROTEIN CO-INGESTION ON TIME TO EXHAUSTION.....	74
<b>2.6 ENDURANCE TRAINING ADAPTATIONS .....</b>	<b>80</b>
2.6.1 CARDIOVASCULAR TRAINING ADAPTATIONS .....	80
2.6.2 INTRAMUSCULAR TRAINING ADAPTATIONS .....	82
2.6.3 PROTEIN INGESTION AND ENDURANCE TRAINING ADAPTATIONS .....	84
<b>2.7 SUMMARY.....</b>	<b>89</b>
<b>CHAPTER 3 .....</b>	<b>91</b>
<b>GENERAL METHODS.....</b>	<b>91</b>
<b>3.1 INTRODUCTION .....</b>	<b>91</b>
<b>3.2 TRIAL OBJECTIVES .....</b>	<b>91</b>
<b>3.3 TRIAL DESIGN .....</b>	<b>94</b>
<b>3.4 PARTICIPANTS .....</b>	<b>96</b>
<b>3.5 ANTHROPOMETRY .....</b>	<b>97</b>
<b>3.6 PRELIMINARY MEASUREMENTS.....</b>	<b>98</b>
3.6.1 RUNNING ECONOMY AND MAXIMAL OXYGEN UPTAKE .....	98
3.6.2 FAMILIARISATION.....	99
<b>3.7 PHYSIOLOGICAL MEASUREMENTS.....</b>	<b>100</b>
3.7.1 URINE OUTPUT.....	100
3.7.2 EXPIRED GAS SAMPLING.....	100
3.7.3 BLOOD SAMPLING.....	102
3.7.4 BLOOD ANALYSIS .....	103
3.7.4.1 Plasma glucose, non-esterified fatty acids, lactate, and urea.....	103
3.7.4.2 Serum insulin .....	103
<b>3.8 MUSCLE BIOPSY PROCEDURES.....</b>	<b>103</b>
3.8.1 MUSCLE BIOPSY SAMPLING .....	103
3.8.2 MUSCLE BIOPSY ANALYSIS.....	105
3.8.3 DECONTAMINATION PROCEDURES .....	106
<b>3.9 SUBJECTIVE MEASUREMENTS.....</b>	<b>107</b>



<b>3.10 CONTROL MEASURES.....</b>	<b>108</b>
3.10.1 STANDARDISATION OF LIFESTYLE.....	108
3.10.2 ENVIRONMENTAL MEASUREMENTS.....	108
<b>3.11 RELIABILITY MEASUREMENTS .....</b>	<b>109</b>
<b>3.12 STATISTICAL ANALYSIS .....</b>	<b>109</b>
<b>CHAPTER 4 .....</b>	<b>112</b>
<b>IMPACT OF MUSCLE GLYCOGEN AVAILABILITY ON THE CAPACITY FOR REPEATED EXERCISE IN MAN.....</b>	<b>112</b>
<b>INTRODUCTION.....</b>	<b>112</b>
<b>MATERIALS AND METHODS.....</b>	<b>114</b>
PARTICIPANTS .....	114
EXPERIMENTAL DESIGN .....	114
EXPERIMENTAL PROTOCOL .....	115
SOLUTION COMPOSITION.....	117
STATISTICAL ANALYSIS.....	120
<b>RESULTS.....</b>	<b>120</b>
EXERCISE CAPACITY .....	120
MUSCLE GLYCOGEN .....	121
PLASMA GLUCOSE AND SERUM INSULIN .....	123
PLASMA NEFA AND LACTATE .....	125
PLASMA UREA .....	125
SUBSTRATE METABOLISM .....	128
HYDRATION AND SUBJECTIVE DATA.....	130
<b>DISCUSSION.....</b>	<b>135</b>
<b>CHAPTER 5 .....</b>	<b>140</b>
<b>INFLUENCE OF POST-EXERCISE PROTEIN INGESTION ON MUSCLE GLYCOGEN METABOLISM IN RECOVERY AND SUBSEQUENT RUNNING EXERCISE.....</b>	<b>140</b>
<b>INTRODUCTION.....</b>	<b>140</b>
<b>MATERIALS AND METHODS.....</b>	<b>142</b>
PARTICIPANTS .....	142
EXPERIMENTAL DESIGN .....	143
EXPERIMENTAL PROTOCOL .....	143

SOLUTION COMPOSITION .....	145
STATISTICAL ANALYSIS.....	147
<b>RESULTS.....</b>	<b>147</b>
EXERCISE CAPACITY .....	147
MUSCLE GLYCOGEN .....	148
PLASMA GLUCOSE AND SERUM INSULIN .....	150
PLASMA NEFA AND LACTATE .....	152
PLASMA UREA .....	152
SUBSTRATE METABOLISM .....	155
HYDRATION AND SUBJECTIVE DATA.....	157
<b>DISCUSSION.....</b>	<b>162</b>
<b>CHAPTER 6 .....</b>	<b>167</b>
<b>RELIABILITY OF TIME TO EXHAUSTION TREADMILL RUNNING AS A MEASURE OF HUMAN ENDURANCE CAPACITY .....</b>	<b>167</b>
<b>INTRODUCTION .....</b>	<b>167</b>
<b>MATERIALS AND METHODS.....</b>	<b>168</b>
PARTICIPANTS .....	168
PRELIMINARY MEASUREMENTS .....	168
EXPERIMENTAL DESIGN .....	169
EXPERIMENTAL PROTOCOL .....	169
STATISTICAL ANALYSIS.....	170
<b>RESULTS.....</b>	<b>171</b>
<b>DISCUSSION.....</b>	<b>178</b>
<b>CHAPTER 7 .....</b>	<b>182</b>
<b>IMPACT OF POST-EXERCISE PROTEIN INGESTION ON ENDURANCE TRAINING ADAPTATION.....</b>	<b>182</b>
<b>INTRODUCTION .....</b>	<b>182</b>
<b>MATERIALS AND METHODS.....</b>	<b>184</b>
APPROACH TO RESEARCH QUESTION.....	184
EXPERIMENTAL DESIGN .....	184
PARTICIPANTS .....	187
NUTRITIONAL SUPPLEMENTS .....	187
BASELINE TESTING.....	189

TRAINING INTERVENTION .....	189
FOLLOW-UP TESTING.....	190
ANTHROPOMETRY .....	190
SUB-MAXIMAL AND MAXIMAL OXYGEN UPTAKE MEASUREMENTS .....	191
HYDRATION.....	191
EXPIRED GAS SAMPLING.....	191
BLOOD SAMPLING .....	192
MUSCLE BIOPSY SAMPLING.....	192
MUSCLE TISSUE SAMPLE PROCESSING .....	193
QUANTITATIVE REAL-TIME PCR.....	193
STANDARDISATION OF LIFESTYLE.....	194
STATISTICAL ANALYSIS.....	195
<b>RESULTS.....</b>	<b>195</b>
PARTICIPANT CHARACTERISTICS .....	195
MAXIMAL OXYGEN UPTAKE.....	198
PLASMA VOLUME AND BLOOD CONSTITUENTS .....	198
INTRAMUSCULAR PARAMETERS .....	201
DIET ANALYSIS.....	201
<b>DISCUSSION.....</b>	<b>203</b>
<b>CHAPTER 8 .....</b>	<b>208</b>
<b>GENERAL DISCUSSION .....</b>	<b>208</b>
8.1 INTRODUCTION .....	208
8.2 MUSCLE GLYCOGEN AVAILABILITY AND THE CAPACITY FOR REPEATED EXERCISE ...	208
8.3 THE ADDITION OF PROTEIN TO CARBOHYDRATE AND MUSCLE GLYCOGEN METABOLISM DURING RECOVERY AND SUBSEQUENT EXERCISE .....	209
8.4 RELIABILITY OF TIME TO EXHAUSTION AS A MEASURE OF HUMAN ENDURANCE CAPACITY .....	210
8.5 POST-EXERCISE PROTEIN INGESTION AND ENDURANCE TRAINING ADAPTATION.....	212
8.6 SUMMARY.....	212
8.7 FUTURE DIRECTIONS .....	216
8.8 CONCLUSIONS .....	219
<b>REFERNECES .....</b>	<b>220</b>
<b>APPENDICES .....</b>	<b>264</b>

## LIST OF TABLES

### CHAPTER 2

Table 2.1 Effects of carbohydrate ingestion during short-term recovery on repeated exercise capacity.	73
---	----

Table 2.2 Effects of carbohydrate-protein ingestion during short-term recovery on repeated exercise capacity.	79
---	----

### CHAPTER 3

Table 3.1 Nutritional composition of the supplements provided in Chapters 4 and 5.	95
--	----

Table 3.2 Coefficient of variation of different parameters measured throughout the experiment chapters.	109
---	-----

### CHAPTER 4

Table 4.1 Substrate metabolism and respiratory exchange ratio (RER) during Run-1 and Run-2 with L-CHO or H-CHO treatments.	132
--	-----

Table 4.2 Relative exercise intensities ( $\% \dot{V}O_{2\max}$ ), ratings of perceived exertion (RPE) and heart rate during Run-1 and Run-2 in L-CHO and H-CHO treatments.	133
---	-----

Table 4.3 Subjective measures of gut fullness, thirst and stomach discomfort following the ingestion of L-CHO and H-CHO.	134
--	-----

### CHAPTER 5

Table 5.1 Substrate metabolism and respiratory exchange ratio (RER) during Run-1, recovery and Run-2 with CHO or CHO-P treatments.	159
--	-----

Table 5.2 Relative exercise intensities (% $\dot{V}O_{2\max}$ ), ratings of perceived exertion (RPE) and heart rate during Run-1 and Run-2 in CHO and CHO-P treatments.	160
---	-----

Table 5.3 Subjective measures of gut fullness, thirst and stomach discomfort following the ingestion of CHO and CHO-P.	161
--	-----

## CHAPTER 6

Table 6.1 Percent (%) $\dot{V}O_{2\max}$ , RPE and heart rate responses to the first and second run times to exhaustion.	173
--	-----

Table 6.2 Substrate metabolism and respiratory exchange ratio (RER) in the first and second run times to exhaustion.	174
--	-----

## CHAPTER 7

Table 7.1 Nutritional composition of the supplements.	188
---	-----

Table 7.2 Participant characteristics pre and post-training.	197
--	-----

Table 7.3 Plasma volume and blood constituents before and after six weeks of training.	200
--	-----

# LIST OF FIGURES

## CHAPTER 2

- Figure 2.1** Regulation of glycogen phosphorylase during exercise. 25
- Figure 2.2** Diagram representing tissue contributions to systemic lactate turnover in healthy humans during exercise (adapted from van Hall, 2010). This diagram only provides an estimate as a result of differences in exercise intensity, durations and tracer use between studies amalgamated to reflect lactate turnover during exercise. 30
- Figure 2.3 Regulation of glucose uptake during/following exercise. Solid arrows represent feed-forward mechanisms and dashed arrows represent feed-back mechanisms. 51
- Figure 2.4 Reported rates of muscle glycogen resynthesis across 17 different investigations that have measured muscle glycogen concentrations during short-term (2-6 h) recovery with varied rates of carbohydrate with or without protein in humans. 67
- Figure 2.5 Flow diagram illustrating the influence of post-exercise protein ingestion during short-term recovery. 87
- Figure 2.6 Flow diagram illustrating the influence of post-exercise protein ingestion on endurance training adaptation. 88

## CHAPTER 4

- Figure 4.1 A schematic representation of the study protocol. 119
- Figure 4.2-A. Muscle glycogen concentrations at the end of Run-1 (F1), at the end of 4 h recovery, time to exhaustion with L-CHO treatment (F2) and time to exhaustion with H-CHO treatment (F3). Figure 4.2-B, mean and individual run times to exhaustion following the ingestion of L-CHO or H-CHO during 4 h recovery. 122

Figure 4.3. Plasma glucose and serum insulin concentrations during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments.	124
Figure 4.4 Plasma NEFA and lactate concentrations during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments.	126
Figure 4.5 The contribution of muscle glycogen, extra-muscular carbohydrate (CHO) and lipids to total substrate metabolism ( $\text{kJ} \cdot \text{min}^{-1}$ ) during Run-2 with L-CHO or H-CHO treatments.	127
Figure 4.6 Plasma urea during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments.	129
Figure 4.7 Short form profile of mood state (POMS-SF) categories recorded before exercise with L-CHO and H-CHO treatments.	131

## CHAPTER 5

Figure 5.1 A schematic representation of the study protocol.	146
Figure 5.2-A Muscle glycogen concentrations at the end of Run-1 (Fatigue 1), at the end of 4 h recovery, and the end of Run-2 (Fatigue 2) with CHO and CHO-P treatments. Figure 5.2-B, mean and individual run times to exhaustion during Run 2 following the ingestion of CHO or CHO-P during 4 h recovery.	149
Figure 5.3 Plasma glucose and serum insulin concentrations during Run-1, recovery and Run-2 with CHO or CHO-P treatments.	151
Figure 5.4 Plasma NEFA and lactate concentrations during Run-1, recovery and Run-2 with CHO or CHO-P treatments.	153
Figure 5.5 The contribution of muscle glycogen, extra-muscular carbohydrate (CHO) and lipids to total substrate metabolism ( $\text{kJ} \cdot \text{min}^{-1}$ ) during Run-2 with CHO or CHO-P.	154
Figure 5.6 Plasma urea during Run-1, recovery and Run-2 with CHO or CHO-	156

P treatments.

Figure 5.7 Short form profile of mood state (POMS-SF) categories recorded before exercise with L-CHO and H-CHO treatments.	158
--	-----

## **CHAPTER 6**

Figure 6.1 Run times compared between trials during walk-1, walk-2 and time to exhaustion.	175
--	-----

Figure 6.2 Scatterplot showing the relationships between the two run times to exhaustion. Straight lines represent best fit for walk-1, walk-2 and time to exhaustion.	176
--	-----

Figure 6.3 Bland-Altman plot of absolute agreement between two run times to exhaustion.	177
---	-----

## **CHAPTER 7**

Figure 7.1 Flow diagram of the study.	185
---------------------------------------	-----

Figure 7.2. Schematic representation of the experimental protocol.	186
--	-----

Figure 7.3 Absolute and relative change in maximal oxygen uptake at baseline and follow-up in CHO and CHO-P groups.	199
---	-----

Figure 7.4 Relative gene expression represented as fold change from baseline for several key genes related to mitochondrial biogenesis, muscle protein synthesis and carbohydrate/lipid metabolism.	202
---	-----

## **CHAPTER 8**

Figure 8.1 Flow diagram illustrating the influence of post-exercise protein ingestion during short-term recovery.	214
---	-----



Figure 8.2 Flow diagram illustrating the influence of post-exercise protein ingestion on endurance training adaptation.	215
Figure 8.3 Reported carbohydrate intakes during 4 h recovery and repeated running exercise capacity in Chapter 4 of the thesis (0, 0.3 and 1.2 g·kg BM <sup>-1</sup> ·h <sup>-1</sup> ) and another study (Betts et al. (2007); 0.8 and 1.1 g·kg BM <sup>-1</sup> ·h <sup>-1</sup> ).	217

## CHAPTER 1

### INTRODUCTION

Athletes across the myriad range of sports are required to participate in a number of vigorous competitive events interspersed with intense and frequent training sessions and minimal time to recover. Collectively, this congested annual schedule imposes significant loads on their physiological and metabolic systems close to the threshold of exhaustion, from which they are required to recover rapidly in preparation for the subsequent exercise bout (Reilly and Ekblom, 2005). In addition, habitually active participants strive to improve their training regime, and improved recovery following each exercise bout can promote their exercise capacity and adherence to partaking in exercise.

Exercise-induced fatigue is a common sensation experienced by any individual undertaking physical exercise. Fatigue during exercise occurs simultaneously at several loci within the neuromuscular system as well as the internal environment (Ament and Verkerke, 2009). Thus, a multitude of mechanisms have been proposed to explain fatigue, ranging from metabolic disturbances in the motor unit to centrally-mediated perturbations (Enoka and Duchateau, 2008; Marino *et al.*, 2011) and thus—albeit reductionist—fatigue can be broadly characterised as peripheral fatigue and central fatigue (Marino *et al.*, 2011). In accordance, research into fatigue is highly complex and a consensus about the aetiology of this phenomenon remains elusive. Not surprisingly, there are numerous definitions of exercise-induced fatigue, as experimentally inducing fatigue is likely to be inherently variable depending the type/duration of exercise and the tools used to assess this phenomenon (Phillips, 2015). In the context of the experimental chapters in this thesis, however, the term ‘fatigue’ or ‘exhaustion’ are used to denote the inability to sustain running speed at a prescribed intensity as indicated by the participant.

While muscle fatigue can be quantified as the decline in maximal force capacity of the muscle due to decrements in the activation signal or the function of the contractile proteins, the mechanisms responsible for this reduction in maximal force capacity depend on the details of the task that is being performed (Gandevia, 2001). Critical task variables include the type/intensity/duration of exercise, the muscle groups involved and the environment in which the task is performed (Maluf and Enoka, 2005). Therefore, a useful strategy to examine muscle fatigue is to identify the mechanisms responsible for the failure specific tasks (Enoka and Duchateau, 2008). Therefore, the mechanisms responsible for task failure during whole-body prolonged submaximal endurance exercise are the central focus in this thesis when discussing fatigue.

Exercise capacity/performance and recovery from exercise can be enhanced by evidence-based nutritional interventions through manipulation of different nutritional variables (i.e. nutrient composition, quantity, timing of nutrient ingestion etc.) (Rodriguez *et al.*, 2009). For several decades, it has been recognised that carbohydrate availability is critical for exercise capacity (Bergstrom *et al.*, 1967; Coyle *et al.*, 1986). This is emphasised during prolonged moderate to high-intensity exercise where the reliance on endogenous carbohydrate stores becomes increasingly important relative to lower intensity exercise (Coyle *et al.*, 1997; Hermansen *et al.*, 1967). Studies in humans clearly demonstrate that fatigue during a prolonged exercise bout coincides with low muscle glycogen content (Bergstrom *et al.*, 1967; Hermansen *et al.*, 1967), and the ingestion of carbohydrate is causally related to the maintenance of performance in humans (Karelis *et al.*, 2010) either by the attenuation of glycogenolysis (Tsintzas and Williams, 1998) or the maintenance of euglycaemia and carbohydrate oxidation (Coyle *et al.*, 1986; Jentjens *et al.*, 2004). Therefore, nutritional interventions that increase pre-exercise intramuscular glycogen stores positively correlate with the capacity for exercise, as muscle glycogen depletion closely parallels perception of fatigue (Bergstrom *et al.*, 1967). Unsurprisingly, this nutritional modulation of exercise has prompted numerous nutritional interventions to target these fatigue mechanisms, leading to general recommendations to optimise muscle glycogen availability (Jeukendrup and Jentjens, 2000; Tsintzas and Williams, 1998). Indeed, both maximising muscle glycogen content prior to exercise and/or

sparing its utilisation during exercise can influence endurance capacity (Bergstrom *et al.*, 1967; Tsintzas *et al.*, 1996b).

In contrast to the above knowledge, there has been less attention with regards to post-exercise nutrition, notwithstanding that recovery is a critical part of training and repletion of muscle glycogen stores is likely to influence the quality of the subsequent exercise bout. Based on the aforementioned nutritional guidelines, it is logical to assume that repeated exercise capacity following short-term recovery would be dictated primarily by the availability of muscle glycogen. Whereas numerous studies have indicated that muscle glycogen restoration is improved with carbohydrate supplementation (for review see (Betts and Williams, 2010)), very few were instigated to examine muscle glycogen degradation during a subsequent bout (Berardi *et al.*, 2006; Betts *et al.*, 2008; Tsintzas *et al.*, 2003). Surprisingly, there is little evidence to support that recommendations aimed to increase muscle glycogen synthesis after an initial exercise bout could influence subsequent exercise capacity just hours later (Betts and Williams, 2010) and thus the current understanding of the importance of glycogen during a repeated exercise bout following short-term recovery remains unclear.

Notwithstanding this, more knowledge can be obtained when addressing the effects of nutrient intake on muscle glycogen repletion or restoration of exercise capacity independently. For example, it has been established that ingesting carbohydrate during recovery augments muscle glycogen resynthesis when compared to placebo (Ivy *et al.*, 1988b; Pascoe *et al.*, 1993). In addition, an exponential relationship was shown between the quantity of carbohydrate ingested and level of muscle glycogen restoration until reaching the maximal threshold for glycogen resynthesis, which is achieved by ingesting carbohydrate at a rate of 1.2 grams per kilogram of body mass per hour ( $\text{g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) during a 5 h recovery period (Burke *et al.*, 2004; van Loon *et al.*, 2000b). The restoration of muscle glycogen has been proposed to occur in two phases; an insulin independent and insulin dependent phase (Beelen *et al.*, 2010; Jentjens and Jeukendrup, 2003). Therefore, many studies were conducted to evaluate the efficacy of mixed macronutrient supplements, specifically carbohydrate-protein

ingestion, given the synergistic effect of these two nutrients on insulin secretion (Rabinowitz *et al.*, 1966; van Loon *et al.*, 2000a). Several of these investigations indicated that increasing carbohydrate intake would be sufficient to maximise glycogen resynthesis and would negate any additional benefit from the inclusion of protein (Beelen *et al.*, 2011; Jentjens *et al.*, 2001; van Hall *et al.*, 2000). Nonetheless, other studies observed an increase in glycogen resynthesis rates, indicating a distinct advantage with protein co-ingestion even when both supplements were matched in their energy content (Berardi *et al.*, 2006; Ivy *et al.*, 2002). Therefore, there is clearly a need for further investigations, particularly running-based exercise modalities, to assess whether protein co-ingestion would enhance post-exercise muscle glycogen restoration.

In relation to subsequent endurance capacity, it has been demonstrated that ingesting carbohydrate ( $0.5 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) during short-term recovery is advantageous in restoring subsequent exercise bout as opposed to a placebo (Fallowfield *et al.*, 1995). Nevertheless, only three studies investigated whether the linear relationship between the amount of carbohydrate ingested and muscle glycogen replenishment would translate into an improvement in subsequent endurance capacity (Betts *et al.*, 2007; Fallowfield and Williams, 1997; Wong and Williams, 2000), with only one indicating the presence of a dose-response relationship (Betts *et al.*, 2007). Nevertheless, there is some evidence to suggest that the restoration of muscle and liver glycogen stores is important in restoring endurance capacity following short-term recovery (Casey *et al.*, 2000). Conversely, repeated exercise capacity/performance was also shown to dissociate from glycogen resynthesis in some circumstances (Berardi *et al.*, 2006; Betts *et al.*, 2007; Betts *et al.*, 2008; Ferguson-Stegall *et al.*, 2011b). Therefore, the available knowledge does not negate the possibility that muscle glycogen availability during repeated exercise may be of less importance and/or may not be the likely cause of fatigue during a subsequent bout. It is interesting to note that subsequent endurance capacity has been improved across a wide range of studies when carbohydrate-protein was ingested (Ferguson-Stegall *et al.*, 2011b; Lunn *et al.*, 2012; Williams *et al.*, 2003). However, the exact mechanism behind this ergogenic effect of protein addition to carbohydrate remains to be elucidated, and thus plausible mechanistic effects to

support the inclusion of protein to carbohydrate during short-term recovery and/or endurance capacity requires further investigation.

Beyond the acute recovery period, the inclusion of protein following prolonged exercise is required for other recovery-related process such as tissue repair/reconditioning. Given the current understanding that nutrient availability can modulate the adaptive response to an acute exercise bout, it is somewhat surprising that very few investigations examined the role of post-exercise protein intake on endurance training adaptations. Therefore, there is outstanding need to extend our understanding about the role of post-exercise nutrition and endurance training adaptation.

In light of these inconsistencies and the small number of running based investigations in this area, the series of studies that comprise this thesis were conducted to address the various issues that remain equivocal regarding optimal macronutrient composition and quantity during post-exercise recovery and repeated exercise. **Chapter 4** was undertaken to establish whether the amount of carbohydrate during a 4 h recovery would present a dose-dependent relationship in relation to muscle glycogen availability and to explore the restoration of endurance capacity and muscle glycogen metabolism during repeated exercise. **Chapter 5** was aimed to address the effects of carbohydrate-protein ingestion as opposed to an isocaloric carbohydrate solution to determine the influence of nutrient composition upon muscle glycogen repletion during short-term recovery and restoration of endurance capacity. The study also examined muscle glycogen metabolism during the repeated exercise bout to elucidate any protein-mediated effects on muscle glycogen. In order to assess the reliability of the chosen outcome measure of fatigue, **Chapter 6** examined the reliability of submaximal treadmill run time to exhaustion to assess human endurance capacity in aerobically-trained men and women. The final study of this thesis (**Chapter 7**) reports the effects of six weeks of post-exercise carbohydrate-protein ingestion in amplifying the adaptive response to running-based endurance training.

## CHAPTER 2

### REVIEW OF LITERATURE

#### 2.1 Substrate metabolism during prolonged endurance-type exercise

##### 2.1.1 Muscle glycogen metabolism during prolonged exercise

It has been known for decades that carbohydrates and fat are the primary substrates for energy metabolism in humans during prolonged exercise; and a shift from the reliance on fat to carbohydrate stores during muscular work was observed with increasing work intensity measured by analysis of respiratory quotient (RQ) (Christensen and Hansen, 1939). These previous estimations did not include any direct measurements of intramuscular substrate utilisation until the reintroduction of the needle muscle biopsy technique in the late 1960s (Bergstrom *et al.*, 1967; Bergstrom and Hultman, 1966; Hermansen *et al.*, 1967) and subsequent non-invasive methods such as magnetic resonance spectroscopy (Krssak *et al.*, 2000) and contemporary isotope methodologies (van Loon *et al.*, 2001). The culmination of human metabolism assessment techniques allowed for a greater knowledge of the relative contribution of these endogenous stores to energy provision and the several factors that may influence metabolism such as, diet, sex, fibre types, training status and the duration, intensity and mode of exercise (Essen, 1977; Romijn *et al.*, 1993; Tarnopolsky *et al.*, 1990; van Loon *et al.*, 2001).

In humans, carbohydrate is stored as glycogen (Johnson *et al.*, 2004) and skeletal muscle represents the most abundant carbohydrate store (Jue *et al.*, 1989; Shulman *et al.*, 1990). The predominant disposition of endogenous carbohydrate stores is located within skeletal muscle (79 %) (Sherman, 1995). Under normal conditions, glycogen content in the muscle ranges between 300-400 mmol glucosyl units·kg dry mass<sup>-1</sup> (Essen, 1977), and training and diet manipulation seem to evoke an increase in muscle glycogen content to about 700-900 mmol glucosyl units·kg dry mass<sup>-1</sup> (Sherman *et al.*, 1981). The breakdown of muscle glycogen is dependent on the activation of

glycogen phosphorylase and debranching enzyme, which ultimately cleave glucose residues from the glycogen chain (Melendez *et al.*, 1999). A number of factors influence the activation of glycogen phosphorylase during exercise and thus allow for a close regulation of glycogenolysis and oxidation with the different energetic requirements of the working muscle (Greenberg *et al.*, 2006). Glycogen phosphorylase is activated by allosteric binding of AMP and IMP so that the enzyme is responsive to the energy state of the cell (Johnson, 1992). Muscular contraction also increases cytosolic calcium ( $\text{Ca}^{2+}$ ) release and an adrenaline-mediated increase in cyclic AMP (cAMP), thereby activating phosphorylase kinase (PK) and the resultant activation of glycogen phosphorylase (Brushia and Walsh, 1999; Watt *et al.*, 2001).

The rate of muscle glycogen degradation is closely related to the intensity and duration of exercise, that is it increases with the former and is often decreased with the latter (Graham *et al.*, 2001). Another important factor for the utilisation of glycogen is the availability of this substrate prior to exercise (Arkinstall *et al.*, 2004), with elevations in the rate of degradation of glycogen during exercise being exponentially related to pre-exercise muscle glycogen concentrations (Hargreaves *et al.*, 1995; Wojtaszewski *et al.*, 2003). In support of this, increasing the glycogen content in one leg was shown to accelerate glycogenolysis twofold compared to the control leg with low glycogen content, which is likely ascribed to a greater increase in the activity of glycogen phosphorylase concomitant to its greater binding to the glycogen granule (Shearer *et al.*, 2001). Thus, the availability of glycogen prior to exercise may modulate its metabolism during subsequent exercise (Hargreaves, 2004).

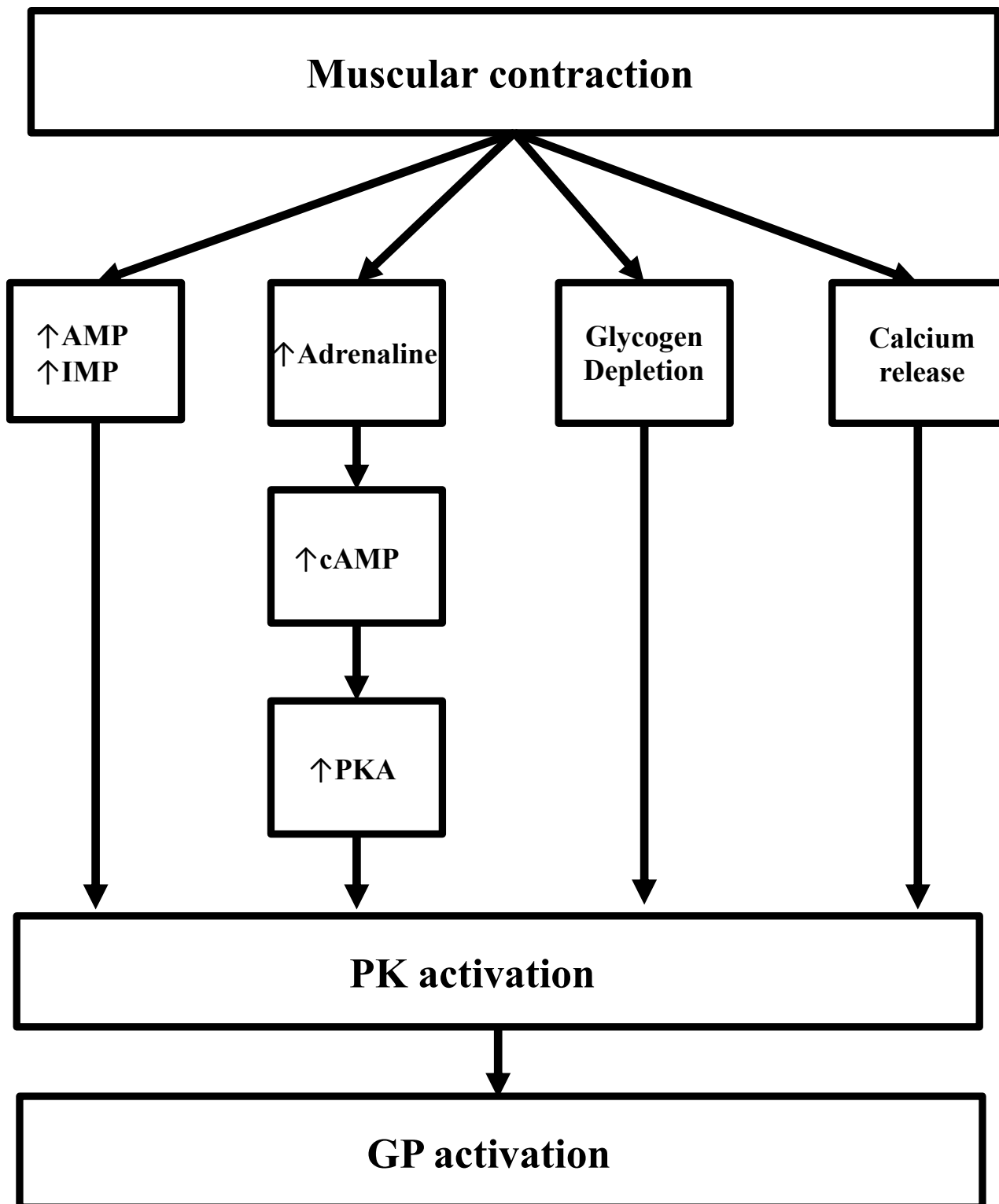
While it is clear that muscle glycogen metabolism is profoundly influenced by substrate availability and exercise intensity, the mode of exercise and whether or not carbohydrate was ingested during exercise seem to be interrelated determinants of equal importance on the utilisation of muscle glycogen during physical work (Tsintzas and Williams, 1998). For example, it was shown that when carbohydrate was ingested during constant pace running, a greater maintenance of blood glucose oxidation such that a glycogen sparing effect was observed and was attributed to a



greater maintenance of carbohydrate oxidation from the exogenous carbohydrate source (Tsintzas *et al.*, 1995) in particular type I muscle fibres (Tsintzas *et al.*, 1996b). In contrast, the majority of cycling based investigations seem to suggest that this phenomenon is not the likely cause of the performance benefits when carbohydrate is ingested (Tsintzas and Williams, 1998), albeit glycogen sparing was also observed during the first hour of cycling when fed carbohydrate (Stellingwerff *et al.*, 2007). Rather, an enhanced maintenance of euglycaemia towards the end of exercise (Coyle *et al.*, 1986) and a suppression of endogenous glucose production (Jeukendrup *et al.*, 1999) were indicated to be the likely mechanisms by which exogenous carbohydrate intake improves cycling performance during prolonged exercise. Furthermore, glucose infusion was shown to improve cycling endurance capacity independent of total carbohydrate oxidation or hypoglycaemia, suggesting that maintenance of glycaemia when glycogen stores are low may have an ergogenic effect (Claassen *et al.*, 2005), possibly via a central mechanism whereby compromised bodily carbohydrate availability limits cerebral glucose uptake and thus amplifying perceived effort and reducing motor activation (Dalsgaard, 2006; Nybo *et al.*, 2003). It can therefore be concluded that the ingestion of carbohydrate presents an ergogenic effect during prolonged moderate to high-intensity endurance exercise, and the physiological mechanisms responsible for these observations appear to involve several inter-related factors including: maintenance of euglycaemia and an attenuation of central nervous system fatigue; glycogen sparing; and reduced exercise-induced strain (Cermak and Loon, 2013; Karelis *et al.*, 2010).

There is clearly a multitude of factors that influence muscle glycogen metabolism during physical exercise. Notably, sex differences may play a role in the discrepancies in the utilisation of endogenous carbohydrate stores (Tarnopolsky *et al.*, 1990; Tremblay *et al.*, 2010). Early reports indicated that increasing carbohydrate intake increase glycogen storage to a lesser extent in women when compared to men (Walker *et al.*, 2000). Albeit these observations are likely to be attributable to the difference in total energy intake (Tarnopolsky *et al.*, 2001), more recent evidence reported no sex differences in the ability to synthesise glycogen (McLay *et al.*, 2007). Irrespective of sex, it appears that training status would be expected to dictate the selection of substrate use during exercise as a result of an enhancement in lipid

oxidation and a concurrent improved insulin-stimulated glucose uptake capabilities when compared to untrained individuals (Brooks and Mercier, 1994; Dela *et al.*, 1992; Tarnopolsky, 2008). A wealth of evidence has demonstrated that endurance training influences substrate selection during exercise and thus contributes to the rate of endogenous carbohydrate metabolism during exercise (Hulston *et al.*, 2010; Jeukendrup *et al.*, 1997; Leblanc *et al.*, 2004; Talanian *et al.*, 2010). Nevertheless, it should be noted that large inter-individual differences exist in the capacity to oxidise lipids (Jeukendrup and Wallis, 2005). In a cross sectional study, Venables *et al.* (2005) demonstrated that lean body mass, fat mass, sex,  $\dot{V}O_{2\max}$  and estimated physical activity level can only account for 34 % in the variability in peak fat oxidation and thus the inter-individual variation in fat oxidation remains largely unexplained (Venables *et al.*, 2005). However, it is likely that a degree of this variation can be accounted for by diet, given that altering the diet to a high carbohydrate/low fat or high fat/low carbohydrate can substantially suppress or elevate fat oxidation, respectively (Yeo *et al.*, 2008a).



**Figure 2.1** Regulation of glycogen phosphorylase during exercise.

### 2.1.2 Extra-muscular carbohydrate metabolism during prolonged exercise

Although muscle glycogen plays a central role for energy metabolism during moderate to high intensity exercise, the importance of other extra-muscular carbohydrate sources (i.e. liver glycogen, blood glucose and lactate) is profound when prolonged exercise is performed (Coyle, 2000). The remarkable contribution of these carbohydrate stores with prolonged duration was shown at low ( $\approx 30\% \dot{V}O_{2\max}$ ) exercise intensities, whereby splanchnic glucose production was sufficient to deplete 75 % of the liver glycogen stores (Ahlborg *et al.*, 1974). These two sources are by no means exclusive of each other, and the fact that increased glucose uptake by the working muscle is quantitatively matched by the liver seem to support this notion (Wasserman and Cherrington, 1991). The liver has a central role in blood glucose homeostasis (Coker and Kjaer, 2005), which is in turn critical for supplying glucose to the various tissues in the post-absorptive period (Wahren *et al.*, 1971) and physical exertion (Suh *et al.*, 2007). The importance of the liver during exercise is also underscored by being the only organ that is capable of considerable glucose production and assimilation (Dardevet *et al.*, 2006) and a substantial disposal of the end products of muscle metabolism (Wasserman and Cherrington, 1991).

The liver ensures the maintenance of glycaemia within a tight range under widely divergent physiological conditions through the dynamic equilibrium of hepatic glucose production mechanisms, namely glycogenolysis and gluconeogenesis, by which the relative contribution is determined by the intensity and duration of exercise in addition to the absorptive state of subjects (Wasserman and Cherrington, 1991; Yabaluri and Bashyam, 2010). Under a resting postprandial state, the production of blood glucose relies primarily on glycogenolysis (Nilsson and Hultman, 1973). Nevertheless, the rapid utilisation of hepatic glycogen and the relatively small quantities of this substrate evoke a gradual decline in these stores (Wahren and Ekberg, 2007). This rapid depletion of hepatic glycogen emphasises the importance of gluconeogenesis with duration (Wahren and Ekberg, 2007), as was shown with a 54 % contribution to glucose production via gluconeogenesis following a 14 h fast (Chandramouli *et al.*, 1997). Thus, recent estimations for the contribution of liver glycogenolysis and gluconeogenesis in glucose turnover are approximately equal (50

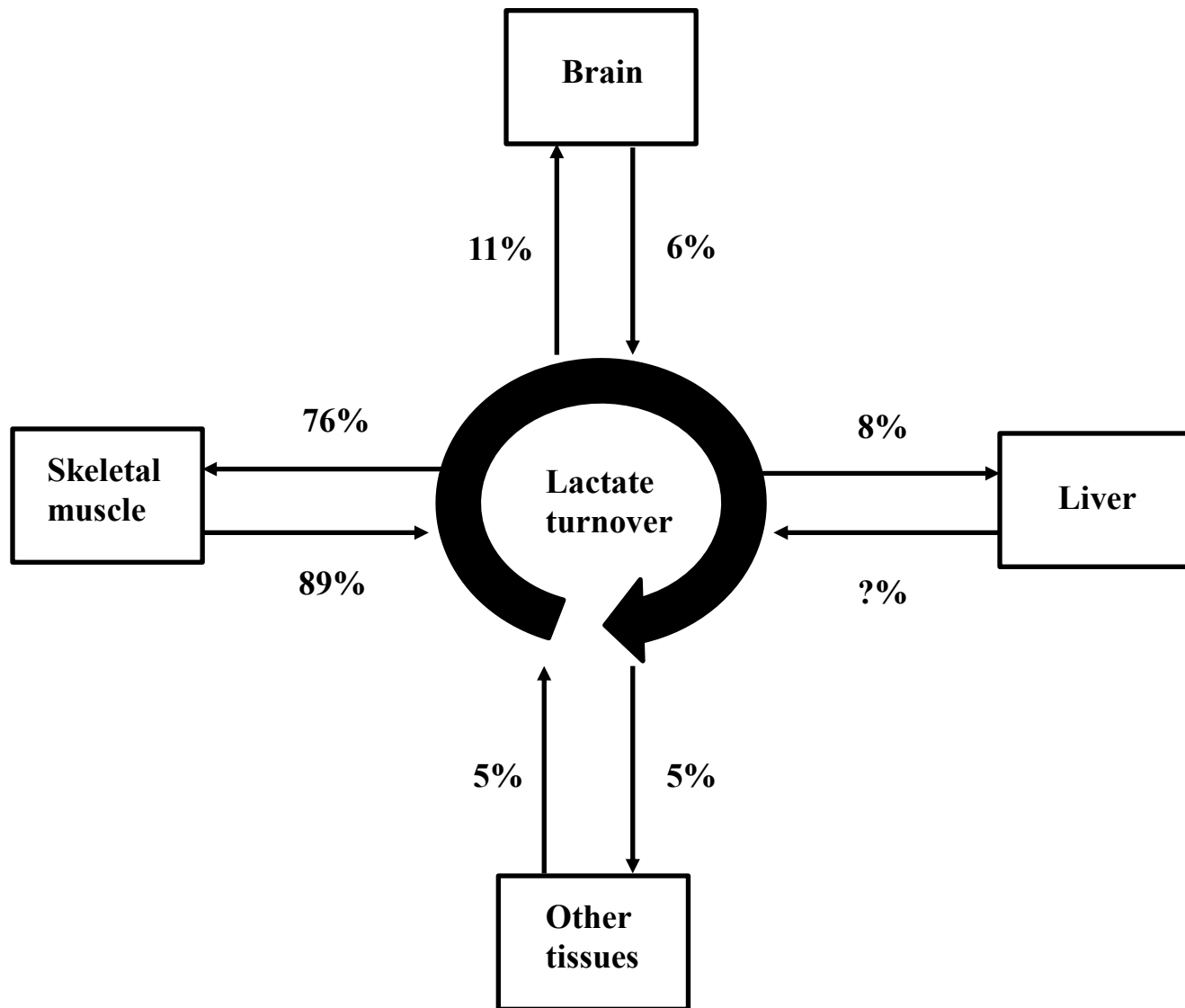
%) in healthy post-absorptive humans (Wahren and Ekberg, 2007). The regulation of hepatic glucose production occurs primarily through circulating insulin levels, despite the fact that basal glucagon levels are required for the stimulation of glucose output in the overnight fasted state (Wasserman *et al.*, 1989). This is evidenced by the modulating influence of insulin on hepatic glycogen degradation in both canines (Sindelar *et al.*, 1996) and humans (Felig and Wahren, 1971), although the sensitivity of glycogenolysis and gluconeogenesis to insulin concentration may differ substantially (Felig and Wahren, 1971). In addition, other indirect insulin-related mechanisms were also observed, such that reductions in lipolysis, muscle proteolysis and glucagon secondary to insulin activity cannot be overlooked (Girard, 2006).

Similarly during exercise, the contribution of both glycogenolysis and gluconeogenesis cannot be overstated in the absence of nutrient (carbohydrate) ingestion. In fact, an appreciable amount (15-30 %) of the energy required for moderate exercise is obtained from blood glucose (Wahren *et al.*, 1971), while supplying most of the fuel for the central nervous system (CNS) (Suh *et al.*, 2007). However, the matching of glucose disposal by the muscle and the appearance of glucose into the circulation during exercise presents a major challenge for this organ (Hoene and Weigert, 2010; Suh *et al.*, 2007). Under postprandial conditions, these complexities during prolonged exercise may be related to the inability of liver glucose output to match the required muscle glucose uptake (Sherman, 1995). This would appear to be an inevitable outcome since the energy requirements for a given exercise intensity remains constant, whereas liver glycogen stores are limited (Wasserman and Cherrington, 1991). During moderate-intensity exercise, the hepatic glucose production was shown to increase threefold from resting conditions (Rowell *et al.*, 1965), which was suggested to be in response to the exercise-induced increments in glucose uptake (Suh *et al.*, 2007). This suggests that hepatic glucose output is the primary extra-muscular source of glucose for the working muscle (Wahren and Ekberg, 2007). It should be noted that although the kidney is capable of producing glucose, its contribution to glucose output during exercise is negligible (Wahren *et al.*, 1971).

Glucose output is almost entirely derived from liver glycogenolysis at the initial stages of prolonged moderate to high-intensity exercise secondary to glucagon and noradrenaline-mediated activation of hepatic glycogen phosphorylase (Suh *et al.*, 2007; Wahren *et al.*, 1971). Indeed, hepatic glycogenolysis is directly related to hepatic glycogen content and thus supporting the notion that glucose output is primarily dependent on liver glycogen content (Vissing *et al.*, 1989). Gluconeogenesis also plays an essential role in maintaining total glucose production to moderate to high-intensity exercise. Indeed, gluconeogenesis was shown to contribute to  $\approx 20\%$  of total glucose production during exercise at  $\approx 70\% \dot{V}O_{2\max}$ , (Emhoff *et al.*, 2013b). The latter study employed tracer methodology to measure glucose flux, which in contrast to venous blood sampling allows accurate partitioning of total glucose production.

Glucoregulation is accomplished by a combination of regulatory controls, namely through feedback (Coker *et al.*, 2002) and feedforward (Kjaer *et al.*, 1991) mechanisms. The concentration of blood glucose plays a central regulatory function, which closely monitors the demands for substrate mobilisation (Suh *et al.*, 2007). In accordance, it was shown that exogenous carbohydrate ingestion mediates a blunting of liver glucose output during exercise (Jeukendrup *et al.*, 1999). Moreover, it was observed that patients with McArdle's disease demonstrate a more pronounced mobilisation of extra-muscular carbohydrate sources during exercise when compared to healthy controls to compensate for the lack of intramuscular glycogenolysis (Vissing *et al.*, 1992). Taken together, blood borne and neural feedback mechanisms appear to exert an important role in the modulation of exercise-induced hepatic glucose output (Coker and Kjaer, 2005). With regards to feedforward mechanisms, it was reported that blood glucose concentrations exhibit a disproportionate increase at the onset of exercise as a result of a rise in blood glucose production from the liver that relatively exceeds glucose uptake (Kjaer *et al.*, 1991). This seems to support a contribution from the central nervous system (CNS) to regulate blood glucose concentration and thus indicate a feedforward governance of glycaemia (Suh *et al.*, 2007). It is understood, nonetheless, that these mechanisms of glucoregulation coexist and that decipherment between them is intricate, given that the experimental isolation of one pathway is likely to be compensated by the other (Suh *et al.*, 2007).

Lactate is an important fuel source which acts a substrate for oxidative metabolism. In fact, the oxidation of blood lactate was shown to contribute 30 % of overall carbohydrate metabolism during continuous moderate to high-intensity (70-75 %  $\dot{V}O_{2\max}$ ) exercise (Emhoff *et al.*, 2013a). During continuous prolonged exercise, most lactate disposal occurs via oxidation in working skeletal muscle, but other tissues such as the brain, liver and kidneys are also involved in lactate turnover (Mazzeo *et al.*, 1986; van Hall, 2010). However, as a glycolytic end product in muscle, lactate is an important precursor for hepatic gluconeogenesis and the production of glucose during exercise, which may subsequently undergo oxidation (Ahlborg *et al.*, 1974; Bergman *et al.*, 2000). Using stable isotope tracer method, it was recently demonstrated that 90 % of lactate was directly oxidised during continuous prolonged exercise at  $\approx 70$  %  $\dot{V}O_{2\max}$ , while 10 % of lactate was indirectly oxidised via gluconeogenesis (Emhoff *et al.*, 2013a). Collectively, the substantial contribution of blood lactate coupled with the contribution of hepatic glycogenolysis and gluconeogenesis is likely to contribute substantially during prolonged moderate to high intensity exercise (Emhoff *et al.*, 2013a, 2013b).



**Figure 2.2** Diagram representing tissue contributions to systemic lactate turnover in healthy humans during exercise (adapted from van Hall, 2010). This diagram only provides an estimate as a result of differences in exercise intensity, durations and tracer use between studies amalgamated to reflect lactate turnover during exercise.



### 2.1.3 Lipid metabolism during prolonged exercise

Aside from carbohydrate, oxidation of fat is the second dominant substrate for aerobic exercise (Sherman, 1995; Spriet, 2002). Although carbohydrate is a preferred substrate during moderate to high-intensity exercise and is likely to determine exercise capacity during such aerobic activities (Coyle, 2000; Stepto *et al.*, 2002), sustained endurance capacity and the attenuation of glycogen depletion are largely mediated by the metabolism of fat by providing fatty acids (FA) for  $\beta$ -oxidation in the mitochondria (Frayn, 2010; Horowitz and Klein, 2000; Wakil and Abu-Elheiga, 2009). The utilisation of fat during high energy demand (i.e. exercise) would appear to be imperative; given that fat is the most abundant endogenous energy depot that is >60 times the amount stored as glycogen in humans, although this is heavily dependent on adiposity (Horowitz and Klein, 2000) whereas carbohydrate availability is limited and therefore is causally related to limitations in the capacity for exercise (Yeo *et al.*, 2011). In accordance, sparing substrate utilisation from finite endogenous carbohydrate stores to fat may be viable to sustain prolonged submaximal exercise (Spriet and Watt, 2003). An intricate interaction exists between carbohydrate and fat metabolism, a number of regulatory mechanisms have been established to explain the inter-play between these two main substrates (for a review see (Hue and Taegtmeyer, 2009)). In concurrence, it is apparent that factors such as intensity and duration of exercise (Perry *et al.*, 2008), training status (van Loon *et al.*, 1999), diet (Yeo *et al.*, 2011) and sex (Cheneviere *et al.*, 2011) have a profound effect on the utilisation of substrates during exercise.

*In vivo* provision of lipids as a substrate for energy metabolism is derived from three sources; triacylglycerol from adipose tissue (TG), intra-myocellular triacylglycerol (IMTG) and circulating plasma triacylglycerol (Kiens, 2006). It is known that the paramount source of FA during exercise is derived from TG whereas the contribution from circulating plasma triacylglycerol is minimal (Jeppesen and Kiens, 2012). Despite the relative small quantities of IMTG when compared to TG, recent understanding suggests that a substantial contribution of this lipid source is utilised during exercise, which may be related to its proximity to the mitochondria (Schrauwen-Hinderling *et al.*, 2006; van Loon, 2004) and its two- to threefold greater availability in type I muscle fibres that exhibit greater fat oxidation capabilities (Shaw

*et al.*, 2010). Nonetheless, controversy still exists on the role of IMTG as a source for  $\beta$ -oxidation that mainly relate to methodological complexities to accurately quantify the contribution of this substrate and the consequent inconstancies of its role during prolonged exercise in the literature (Shaw *et al.*, 2010). With this in mind, a consistent finding was that non-TG fat sources contribute significantly to moderate intensity exercise (Romijn *et al.*, 1993; van Loon, 2004; van Loon *et al.*, 2001).

Triacylglycerol may practically serve as an unlimited fuel source to support the energetic requirements during aerobic activities given their quantitative superiority to be stored over carbohydrate, and their higher energy density than per molecule of glucose (Spriet, 2002). On the other hand, numerous factors exist to limit the reliance on lipids when compared to carbohydrate as a fuel source during prolonged endurance-type exercise. In contrast to carbohydrate oxidation, whereby the glucose production and uptake by the active muscles are sustained provided sufficient glycogen availability (Suh *et al.*, 2007), there is no evidence of such a mechanism to control the availability-utilisation cycle of FA to the energetic requirements of the working muscle (Holloszy *et al.*, 1998). At low to moderate-intensity exercise an 8-10-fold increase in whole-body fat oxidation is observed that is largely derived from TG as a result of its increased availability in circulation, uptake and oxidation by the exercising muscle secondary to  $\beta$ -adrenergic stimulation (Romijn *et al.*, 1993; van Loon *et al.*, 2001). While this enhancement in fat oxidation is presumably linked to a substantial increase in lipolysis and adipose tissue perfusion via increased blood flow (Arner *et al.*, 1990; Spriet, 2002), this relationship does not hold in higher intensities (Frayn, 2010). At moderate- to high intensity exercise ( $\approx 75\% \dot{V}O_{2\max}$ ) the contribution of fat oxidation is blunted by  $\approx 34\%$  when compared with lipid utilisation rates during lower intensities ( $\approx 55\% \dot{V}O_{2\max}$ ) (van Loon *et al.*, 2001). In a similar manner, the oxidation of fat at higher-intensities ( $\approx 85\% \dot{V}O_{2\max}$ ) becomes down-regulated when compared to moderate intensities ( $\approx 65\% \dot{V}O_{2\max}$ ) (Romijn *et al.*, 1995) and appears to dissociate from a decline in plasma non-esterified free fatty acids (NEFA) availability and/or blood flow (van Loon *et al.*, 2001).

A number of factors have been suggested for the down-regulation of fat oxidation at higher exercise intensities. Failure in adipose tissue to supply the exercising muscle with sufficient FA may be related to an inhibition of fat mobilisation or an inadequate perfusion of the adipose tissue (Frayn, 2010). It is known the catecholaminergic response (a primary regulator of lipolysis) increases exponentially with exercise intensity (Galbo, 1983), which mediates an  $\alpha$ -adrenergic inhibition of lipolysis and thus antagonising  $\beta$ -adrenergic stimulation of lipolysis (Castan *et al.*, 1994). The concentration of lactate is also known to increase with exercise intensity (van Loon *et al.*, 2001). Coupled with the knowledge that the lactate receptor G-protein-coupled receptor (GBR81) is expressed in adipose tissue and that this receptor mediates a marked anti-lipolytic action (Liu *et al.*, 2009). Indeed, other hormonal stimuli of lipolysis such as insulin, growth hormone and cortisol may also be involved (Frayn, 2010). Moreover, using positron emission tomography (PET), it was recently demonstrated that the infusion of noradrenaline (an  $\alpha$ -adrenoreceptor agonist) reduced adipose tissue blood flow by  $\approx 40\%$  both at rest during low-intensity single leg exercise (Heinonen *et al.*, 2013). Thus, it is possible that a reduction in adipose tissue blood flow secondary to the elevations in catecholaminergic response with increased exercise intensity may also be involved in the limitations in fatty acid delivery.

When focussing on the limitations of FA oxidation from the transition from moderate to high-intensity exercise, the failure of the muscle to oxidise FA in the mitochondria may also be a candidate. The oxidation of long chain FA have to be converted to their acylcarnitine form to enter the mitochondria for  $\beta$ -oxidation by a reaction catalysed by carnitine palmitoyltransferase 1 (CPT-1) (Jeppesen and Kiens, 2012). Indeed, increasing exercise intensity was shown to result in a decrease in free carnitine pool (Stephens *et al.*, 2007) and CPT-1 activity is dependent on the availability of carnitine (Harris *et al.*, 1987). Thus, the reduction in intra-muscular free carnitine limits the ability of CPT-1 to transport long chain FA to the mitochondria (Roepstorff *et al.*, 2005). Therefore, it is possible the carnitine is a major regulator of FA oxidation with increasing exercise intensity (Jeppesen and Kiens, 2012).

In summary, an important adaptation to endurance training is a shift in proportionate substrate utilisation from carbohydrate towards fat oxidation, mainly to preserve the limited endogenous carbohydrate stores (Spriet and Watt, 2003). This is principally achieved by an augmentation in mitochondrial volume, FA transport and enzymatic adaptations (e.g. increase hydroacyl-CoA-dehydrogenase) to use fat, a reduction of cell signals such as ADP and AMP during exercise at submaximal work rates and thus reducing the activation of key enzymes (e.g. glycogen phosphorylase and pyruvate dehydrogenase) of carbohydrate metabolism (Egan and Zierath, 2013; Talanian *et al.*, 2010). Furthermore, it has been demonstrated that nutritional manipulation (i.e. high-fat diets) may provide an additive but distinct adaptation to augment the rates of fat oxidation (Yeo *et al.*, 2011), although the efficacy of such practices on endurance capacity are equivocal (Vogt *et al.*, 2003) and may have health risk implications (Hawley *et al.*, 2011). The intensity and duration of the exercise bout appear to be crucial factor in selective substrate utilisation (Romijn *et al.*, 1993; van Loon *et al.*, 2001). It has been shown that the maximal rate of fat oxidation occurs at intensities of  $\approx 65\% \dot{V}O_{2\max}$  (Achten and Jeukendrup, 2003), with greater oxidation rates in running than in cycling (Capostagno and Bosch, 2010); although unexplained individual variation to oxidise fat also exist irrespective of training status and sex (Venables *et al.*, 2005). At higher intensities than those suggested to maximise fat oxidation rates, oxidation of fat declines markedly, possibly by the down-regulation of lipolysis via lactate, catecholamine-mediated stimulation of  $\alpha$ -adrenergic receptors and consequently a reduction in adipose tissue blood flow and/or a reduction in the muscle's ability to oxidise FA in the mitochondria secondary to reductions intramuscular free carnitine (Frayn, 2010; Jeppesen and Kiens, 2012).

#### **2.1.4 Protein metabolism during prolonged exercise**

Protein metabolism serves as an auxiliary fuel source during prolonged aerobic exercise by contributing to  $\leq 5\%$  of ATP provision (Tarnopolsky, 2004). Although some estimates postulate that higher percentages of energy metabolism are derived from intra- and extra-muscular protein sources that may reach 20 %, the contribution of amino acids is clearly relatively small in comparison to the predominant fuel sources (i.e. carbohydrate and fat) (Rennie *et al.*, 2006). From a quantitative

standpoint, protein is less important than other substrates to supply energy during prolonged exercise (Gibala, 2001), but by no means is the contribution from protein negligible, as evidenced by the profound alterations in whole body protein metabolism and amino acid kinetics in response to exercise that consequently regulate fuel metabolism and the adaptive response to training (Gibala, 2007; Rennie *et al.*, 2006; Shimomura *et al.*, 2004).

During exercise, the branched-chain amino acids (BCAA) are preferentially oxidised to other forms of amino acids through transamination process to become transferred into keto-acids by the rate limiting enzyme branched-chain oxo-acid dehydrogenase (BCOAD) (Boyer and Odessey, 1991; McKenzie *et al.*, 2000). The activity of BCOAD was shown to be elevated from the transition from rest to exercise, implying an analogous increase in BCAA oxidation by the working skeletal muscle (Gibala, 2007; Wagenmakers, 1989). Studies have corroborated these findings by demonstrating an increase in leucine oxidation during endurance exercise (Lamont *et al.*, 2003; Phillips *et al.*, 1993). The disruption of BCAA metabolism was shown to severely impair endurance exercise to exhaustion within the skeletal muscle in rodents (She *et al.*, 2010). It seems paradoxical therefore that endurance training results in a reduction in leucine oxidation (Gaine *et al.*, 2005). Indeed, it was observed that following endurance training (90 minutes of 65 %  $\dot{V}O_{2\max}$ ) leucine oxidation and BCOAD activation were lower in both males and females (McKenzie *et al.*, 2000). However, an examination of leucine kinetics and BCOAD activation revealed that BCOAD capacity was higher following training; an indicative that the absolute capacity for BCAA oxidation had increased and a concomitant exercise-induced efficiency in protein metabolism (McKenzie *et al.*, 2000; Tarnopolsky, 2004). These findings should be interpreted with caution, as other investigations showed below baseline leucine oxidation levels during 3 h of cycling at 75%  $\dot{V}O_{2\max}$  (Devlin *et al.*, 1990) and that the proportion of protein utilisation during exercise remains largely equivocal, possibly due to methodological limitations (Tipton and Wolfe, 2004).

The role of protein metabolism during exercise has also been linked to the provision of precursors for tricarboxylic acid (TCA) reaction; a major common pathway for the

oxidation of carbohydrate, fats and amino acids (Gibala *et al.*, 1998; Gualano *et al.*, 2011). The oxidation of BCAA may induce a cataplerotic state on TCA cycle intermediates, as the catabolism of BCAA requires the consumption of a specific intermediate from this cycle (2-oxoglutarate), which theoretically could impair TCA cycle flux and hence aerobic energy provision (Bowtell *et al.*, 2007; Gibala, 2007). Data from animal studies appear to support the notion that aerobic energy delivery is unaffected by TCA cycle reductions (Dawson *et al.*, 2005). In humans, a dissociation between TCA cycle pool size and aerobic phosphorylation was demonstrated (Gibala *et al.*, 2002; Howarth *et al.*, 2004), suggesting that TCA intermediate content does not represent any functional importance to oxidative phosphorylation (Bowtell *et al.*, 2007).

## **2.2 Mechanisms of fatigue during prolonged endurance-type exercise**

### **2.2.1 Muscle glycogen depletion during prolonged exercise**

The interest in muscle glycogen as an important fuel source during exercise stemmed from researchers in Scandinavia, who demonstrated that appreciable amounts ( $\approx 33\%$ ) of muscle glycogen were degraded when exercise intensity was relatively light (cycling at 50 Watts) for a duration of 30 min (Bergstrom and Hultman, 1966). This prompted a series of studies to assess the relationship between exercise and glycogen metabolism (Ahlborg *et al.*, 1967; Hermansen *et al.*, 1967). These studies consequently revealed an intimate relationship between muscle glycogen concentrations and exercise capacity, such that the capacity for prolonged exercise is directly correlated to the availability of muscle glycogen in the working muscles. This was corroborated by findings that fatigue coincided with critically low muscle glycogen stores (Bergstrom and Hultman, 1967a). An intuitive assumption to this knowledge is that increasing pre-exercise muscle glycogen would determine the ability to perform prolonged moderate to high intensity exercise. This hypothesis was tested by the one of the most heavily cited studies in this area (Bergstrom *et al.*, 1967). This study confirmed that increasing glycogen content by a three day high-carbohydrate nutritional intervention resulted in a concomitant improvement ( $\approx 32\%$ ) in the capacity to perform prolonged exercise when compared to a normal diet, such

that a positive correlation ( $r = 0.92$ ;  $p < 0.001$ ) was shown between pre-exercise glycogen concentration and time to exhaustion in humans (Bergstrom *et al.*, 1967).

Further support for the importance of glycogen during exercise may also be observed in McArdle's disease, where patients are characterised by considerable impairments in exercise performance as a result of the absence of glycogen phosphorylase (Kitaoka, 2014; Lewis and Haller, 1986). Indeed, the relationship between the onset of fatigue during prolonged physical exertion and depletion of muscle glycogen to critically low levels ( $\leq 100$  mmol glucosyl units.kg  $\text{dm}^{-1}$ ) is a consistent finding in numerous investigations using different exercise protocols (Balsom *et al.*, 1999; Bangsbo *et al.*, 1992; Tsintzas *et al.*, 1996b). The latter clearly demonstrates that muscle glycogen depletion is associated with fatigue across a wide range of endurance-type exercise modes, which was also demonstrated in the earlier studies concerning muscle glycogen (Ahlborg *et al.*, 1967; Bergstrom *et al.*, 1967; Hermansen *et al.*, 1967). Therefore, it is now well-established that fatigue during prolonged endurance exercise is largely dependent on muscle glycogen concentrations, and that the presence of adequate amounts of muscle glycogen is crucial for optimal muscle function (Allen *et al.*, 2008; Nielsen and Ortenblad, 2013). What is less clear, however, is the underlying mechanism linking fatigue with muscle glycogen depletion.

Another established concept in muscle glycogen utilisation is that an increase in exercise intensity results in a concomitant rise in glycogen degradation during exercise (Romijn *et al.*, 1993; van Loon *et al.*, 2001). In relation to the pattern of glycogen depletion in human skeletal muscle, an exponential relationship was observed between the intensity of the exercise performed and the rate of glycogen breakdown (Gollnick *et al.*, 1974). The study of Gollnick *et al.* (1974) also demonstrated that the glycogen utilisation in moderate to high intensity exercise ( $64\% \dot{V}O_{2\text{max}}$ ) seems to occur preferentially in the high oxidative type I muscle fibres, and that progressive utilisation of type II muscle fibres occurs upon depletion of the former. In concordance, other investigations have demonstrated that glycogenolysis occurs primarily in type I muscle fibres during moderate to high-intensity exercise

(Gollnick *et al.*, 1973; Vollestad and Blom, 1985; Vollestad *et al.*, 1984), although there may be some glycogen degradation in type IIa muscle fibres (Vollestad and Blom, 1985). Indeed, recent evidence indicates a preferential subcellular depletion of intra-myofibrillar glycogen than inter-myofibrillar or subsarcolemmal glycogen during prolonged exhaustive exercise; an indicative that type I muscle fibres are utilised preferentially before type II fibres because of their greater content (80 %) of intra-myofibrillar glycogen at rest than type II fibres (Nielsen *et al.*, 2011) and thus highlighting the importance of glycogen content in these fibres. In concurrence, it was shown that glycogen utilisation was by far greater in type I fibres during treadmill running at 70 %  $\dot{V}O_{2\max}$ , and that fatigue coincided with depletion of these fibres (Tsintzas *et al.*, 1996b).

Recent human investigations indicate a close link between localised glycogen depletion and the capacity to perform whole-body exercise. Specifically, a reduction in intra-myofibrillar glycogen levels has been associated with impaired sarcoplasmic reticulum (SR)  $Ca^{2+}$  release (Nielsen *et al.*, 2011), which was also shown in skinned rat muscle fibres (Nielsen *et al.*, 2009). Furthermore, single mouse muscle fibres exhibited fatigue-induced impairment in tetanic  $Ca^{2+}$  release when intra-myofibrillar glycogen were reduced to low levels, suggesting that SR  $Ca^{2+}$  release critically depend on energy supply from the intra-myofibrillar glycogen pool (Nielsen *et al.*, 2014). Interestingly, low muscle glycogen following exhaustive exercise was shown to depress muscle SR  $Ca^{2+}$  release rate and impair work output following 4 h of recovery in elite endurance athletes (Gejl *et al.*, 2014). However, when athletes were provided with carbohydrate ( $1.06 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) during the 4 h recovery period, glycogen concentrations were elevated and SR  $Ca^{2+}$  release rate returned to pre-exercise levels and consequently work output was also normalised. Moreover, an association between glycogen and SR  $Ca^{2+}$  release by manipulating muscle glycogen availability during recovery from fatiguing exercise was demonstrated in cross-country skiers (Ortenblad *et al.*, 2011). Similar to the study of Gejl *et al.* (2014), the authors demonstrated that carbohydrate ingestion resulted in restoration of muscle glycogen and a normalised in SR  $Ca^{2+}$  release, whereas both glycogen and SR  $Ca^{2+}$  release were depressed when carbohydrate was withheld from participants (Ortenblad



*et al.*, 2011). Indeed, a reduced SR  $\text{Ca}^{2+}$  release will *per se* cause a decrease in tetanic intracellular free  $\text{Ca}^{2+}$  (Ortenblad *et al.*, 2013), which is in line with studies demonstrating a faster decrease in tetanic  $\text{Ca}^{2+}$  in fibres with low muscle glycogen (Chin *et al.*, 1997; Helander *et al.*, 2002). Therefore, the notion that muscle glycogen depletion is associated with disruption in SR  $\text{Ca}^{2+}$  release and muscular work may lend an explanation to the intimate relationship between the capacity for exercise and muscle glycogen availability. However, the mechanistic evidence to elucidate how and why glycogen levels impair muscle function remain unclear (Nielsen and Ortenblad, 2013; Ortenblad *et al.*, 2013).

### **2.2.2 Hepatic glycogen depletion and hypoglycaemia during prolonged exercise**

Although the consensus view is that initial muscle glycogen content is a critical determinant of exercise capacity, other factors must not be overlooked as the onset of fatigue may be the result of other mechanisms irrespective of muscle glycogen depletion (Sahlin *et al.*, 1998). With diminishing muscle glycogen stores during prolonged exercise, the reliance on extra-muscular glucose is increased in an attempt to maintain blood glucose levels to supply the increasing energetic demands of the working muscles (Coyle, 2000). A landmark study by Coyle *et al.* (1986) investigated the effects of exogenous carbohydrate ingestion during prolonged exercise at  $\approx 70\%$   $\dot{\text{V}}\text{O}_{2\text{max}}$  in endurance-trained cyclists (Coyle *et al.*, 1986). In agreement with the well-established ergogenic effect of carbohydrate ingestion during prolonged (> 90 min) exercise, time to exhaustion was extended when carbohydrate was ingested relative to a placebo (242 *versus* 182 min, respectively). Importantly, the postponement of fatigue with carbohydrate feeding was attributed to blood glucose oxidation despite that 170 mmol glucosyl units·kg dry mass<sup>-1</sup> of muscle glycogen was available in the working muscle at the point of volitional exhaustion. It was concluded from these results that muscle glycogen concentrations were substantially depleted during the initial 3 h of cycling to stimulate muscle glucose uptake, and the development of fatigue was associated with hypoglycaemia (Coyle *et al.*, 1986). These findings may explain, at least in part, the ergogenic benefit of carbohydrate ingestion during exercise through prevention of endogenous carbohydrate depletion

(specifically liver glycogen degradation) and maintenance of extra-muscular carbohydrate oxidation (Jeukendrup, 2004; Jeukendrup *et al.*, 1999).

Nonetheless, it was subsequently demonstrated that muscle glycogen content does not influence the rate of muscle glucose disposal during exercise, rather the availability of blood glucose in circulation primarily regulates muscle glucose disposal under such conditions (Arkinstall *et al.*, 2004; Weltan *et al.*, 1998a, 1998b). Moreover, evidence using glucose infusion during exercise demonstrates that the ergogenic effect of exogenous carbohydrate is unrelated to changes in carbohydrate oxidation and that fatigue under such circumstances is not a direct consequence of hypoglycaemia (Claassen *et al.*, 2005). The authors speculated that fatigue during prolonged exercise may be related to the depletion of bodily carbohydrate stores (i.e. muscle and liver glycogen), which in turn mediate cerebral sensitivity to reductions in blood glucose concentrations. This suggestion is partially supported by observations of a positive correlation ( $r = 0.55$ ;  $p < 0.05$ ) between both liver and muscle glycogen and the capacity for exercise (Casey *et al.*, 2000) coupled with the knowledge that reduced carbohydrate availability may limit cerebral glucose uptake and thus increase perception of effort and consequently impair motor activation (Dalsgaard, 2006; Nybo *et al.*, 2003). Therefore, it is logical to infer that as a consequence of the substantial reliance on carbohydrate as an energy substrate during prolonged exercise, fatigue will often be associated with reduced carbohydrate availability. Despite the fundamental role of muscle glycogen depletion on exercise capacity, interactions with other mechanisms such as liver glycogen depletion and cerebral sensitivity to reduced bodily carbohydrate availability may also be involved.

### **2.2.3 Dehydration and hyperthermia during prolonged exercise**

It is well established that limits to human performance can be mediated by dehydration through an increase in physiological strain and perceived exertion under temperate or warm/hot environments (Cheuvront *et al.*, 2005; Murray, 2007; Shirreffs and Sawka, 2011). This is particularly evident during prolonged exercise training and competition, where dehydration, thermoregulation, electrolyte disturbances and cardiovascular strain usually accompany such activities and may even compromise

health (Von Duvillard *et al.*, 2004). A large body of evidence has consistently demonstrated that dehydration of  $>2$  % BM impairs exercise performance, mainly through exercise-mediated heat stress and subsequent sweat loss, albeit the mechanism by which dehydration contributes to fatigue remains to be debated (Sawka *et al.*, 2011; Sawka and Noakes, 2007; Wall *et al.*, 2013). It appears, nonetheless, that dehydration can impair temperature regulation and aerobic performance independent from and in conjunction with environmental heat stress (Cheuvront *et al.*, 2010).

Decrements in aerobic capacity/performance related to heat-stress and/or dehydration have been evaluated by time to exhaustion or time trial measurements in both laboratory and field settings. A study by Galloway and Maughan (1997) systematically demonstrated the influence on endurance exercise capacity with graded increments with ambient temperature with a range of 4-31 °C. A reduction in the capacity for exercise by approximately 42 min ( $\approx 40$  %) was demonstrated by the authors with the highest relative (i.e. 31 °C) compared to the observed optimal (11°C) temperature (Galloway and Maughan, 1997). It is difficult to establish whether the decrements in exercise capacity in the previous study were related to dehydration and the resultant hyperthermia, given that catecholaminergic response to exercise increases substantially with heat stress (Hargreaves *et al.*, 1996; Parkin *et al.*, 1999) and thus glycogen utilisation rate may be augmented under such circumstances (Febbraio *et al.*, 1994; Jentjens *et al.*, 2002). Nevertheless, follow-up evidence refutes this possibility by demonstrating that fatigue during heat stress was causally related to hyperthermia (Gonzalez-Alonso *et al.*, 1999; Parkin *et al.*, 1999). This observation was shown irrespective of the fact that with increased heat stress, substrate depletion has been shown to accelerate and most notably an augmented muscle glycogen utilisation concurrent with reduced oxidation of ingested exogenous carbohydrate has been observed (Febbraio *et al.*, 1994; Jentjens *et al.*, 2002).

The effects of dehydration and concomitant hyperthermia have also been associated with alterations in the central nervous system (CNS) that are associated with increased core and skin temperatures. Some evidence also supports the notion of a degraded CNS function with hyperthermia based on direct brain wave (EEG) motor-

neural function that is consistent with fatigue (Nybo and Nielsen, 2001) and an accumulation of substrates known to be detrimental to brain function such as hyperammonaemia (Nybo, 2010; Wilkinson *et al.*, 2010). Nevertheless, the metabolic perturbation of hyperthermia appear to be secondary to the effects of thermoregulation and cardiovascular strain, as evidenced by the observation that fatigue during endurance exercise can be instigated long before muscle glycogen depletion can be a contributing factor to the cessation of exercise (Cheuvront *et al.*, 2010; Febbraio, 2001; Galloway and Maughan, 1997). In summary, the production of sweat to maintain thermoregulation is likely to evoke involuntary dehydration even under both warm/hot and temperate environments. The physiological mechanisms contributing this phenomenon include augmented hyperthermia, increased cardiovascular strain and secondary metabolic and central nervous system deteriorations (Sawka and Noakes, 2007). In turn, the physiological homeostatic response to regulate the body's internal temperature and the consequent exacerbated physiological parameters (HR, RPE, core temperature and accelerated glycogenolysis) appears to be associated with impairments in aerobic performance capacity (Cheuvront *et al.*, 2010; Logan-Sprenger *et al.*, 2013). Although dehydration  $>2\%$  BM was consistently established to impair aerobic exercise performance (Sawka and Noakes, 2007), other reports appear to refute these negative effects (Robinson *et al.*, 1995; Slater *et al.*, 2006). However, these findings may be confounded by stomach discomfort during fluid replacement (Robinson *et al.*, 1995) and the use of anaerobic performance measure that only lasted for  $\approx 4$  min (Slater *et al.*, 2006), which in contrast to prolonged aerobic exercise, does not appear to be affected by dehydration (Institute of Medicine, 2005).

#### **2.2.4 Central mechanisms of fatigue during prolonged exercise**

Although the fatigue mechanisms discussed previously are mainly peripheral, exercise performance and fatigue are likely to be determined by a delicate interplay between central and peripheral factors (Nybo and Secher, 2004). Fatigue is known to be a complex phenomenon that may occur at several loci, and a reduced central drive to the contractile process of the muscle may be a candidate in the development of fatigue during endurance exercise as evidenced by the reduced corticospinal drive

while the muscle maintains the ability to contract (Amann *et al.*, 2013; Nybo and Secher, 2004). It has been proposed that central fatigue is instigated by two main factors. The first includes inadequate substrate availability to the brain by which reduced carbohydrate availability (i.e. hypoglycaemia or glycogen depletion) limits cerebral uptake of glucose, exacerbating the perceived effort of exercise and reducing the cerebral metabolic rate that ultimately limits motor activation (Dalsgaard, 2006; Nybo *et al.*, 2003). Interestingly, the uptake of glucose and lactate by the brain is increased relative to oxygen when the brain is activated by exhaustive exercise, and such metabolic changes are influenced by the will to exercise (Dalsgaard *et al.*, 2002).

Under such sustained exercise-induced neuronal activity, brain glycogen may play a bigger role than previously thought such that can be utilised increasingly when glucose supply is limited (Choi *et al.*, 2003). Brain Glycogen is thought to be degraded as a protective mechanism for cerebral function under conditions that compromise glucose homeostasis such as hypoglycaemia (Herzog *et al.*, 2008). Prolonged exhaustive exercise elicits an increase in brain neurotransmitters such as 5-hydroxytryptamine (5-HT) and noradrenaline, which may mediate glycogenolysis within the astrocytes (Brown, 2004; Nybo and Secher, 2004). Recently, Matsui *et al.* (2011) demonstrated that prolonged endurance exercise that induced muscle and liver glycogen depletion was associated with an increase in cerebral noradrenaline and 5-HT, and a consequent brain glycogen depletion by  $\approx 50\%$  (Matsui *et al.*, 2011). Furthermore, it was recently demonstrated that brain glycogen supercompensation was present following exhaustive exercise and the extent of supercompensation preceded that of muscle glycogen (Matsui *et al.*, 2012). Thus, brain glycogen can be an important contributor to energy metabolism where supply transiently cannot meet demand (Gruetter, 2003), such conditions that usually occur during prolonged endurance exercise.

A second interrelated candidate instigating CNS fatigue during prolonged exercise is the synthesis and metabolism of central monoamines, particularly 5-HT which is associated with control of arousal, sleep-wakefulness and mood, hereafter may affect the drive for exercise (Meeusen *et al.*, 2006; Newsholme *et al.*, 1987). Tryptophan is

the precursor for 5-HT synthesis, and the transport of the former across the blood-brain barrier is influenced by plasma concentration of free tryptophan, and other large neutral amino acids (LNAAAs, including the BCAA leucine) that share the same carrier system (Pardridge, 1998). Plasma tryptophan and non-esterified free fatty acids (NEFA) also share albumin as a transporter protein in circulation (Meeusen *et al.*, 2006). Under normal physiological conditions, only 10 % of tryptophan is in free form, while 90 % are bound to albumin, while NEFA are almost completely bound to albumin (Curzon *et al.*, 1973). During prolonged endurance exercise, however, BCAA concentration is reduced via oxidation by the active muscles concurrent with a rise in systemic NEFA levels that occur during such exercise (Shimomura *et al.*, 2004). The latter shift in substrate mobilisation favours the displacement of bound tryptophan from albumin and thus elevates free-tryptophan:BCAA ratio and ultimately increase the synthesis of 5-HT in the brain (Newsholme *et al.*, 1987). While there is some evidence to support the increase in free tryptophan initiates a cascade of events that increase brain 5-HT synthesis (Davis and Bailey, 1997), this hypothesis goes with contention as the association between plasma and brain tryptophan concentration remains to be unequivocally determined (Fernstrom and Fernstrom, 2006).

Indeed, experimental evidence regarding pharmacological manipulation of 5-HT appear to suggest a dose-dependent reduction in exercise capacity with acute administration of serotonin agonist (Bailey *et al.*, 1992). Conversely, an improved exercise capacity was demonstrated when serotonin antagonist was administered (Bailey *et al.*, 1993) and thus endorsing a causal effect between 5-HT and exercise capacity in rodents. Moreover, direct administration of tryptophan into the cerebroventricular region leading the increased 5-HT concentration was associated with a reduced time to exhaustion by 60-70 % (Cordeiro *et al.*, 2014; Soares *et al.*, 2007) and this effect was abolished when an inhibitor of the conversion of tryptophan to 5-HT was injected (Cordeiro *et al.*, 2014). The study by Cordeiro and colleagues (2014) also demonstrated that blocking the exercise-induced conversion of tryptophan to 5-HT did not improve physical exercise, endorsing the notion that other neurotransmitters may also be involved in central fatigue such as dopamine and

noradrenaline (Meeusen *et al.*, 2006) and an overly reductionist view may not be suitable when examining brain neurotransmitters and fatigue.

Most human experiments have been directed towards BCAA and their effects in postponing central fatigue, given the established role of BCAA in brain function (Fernstrom, 2005). Despite the promising rationale, nutritional manipulations to influence central fatigue in humans have been less conclusive. The ingestion of BCAA was shown to reduce perceived exertion and mental fatigue (Blomstrand *et al.*, 1997) and may improve performance during a competitive marathon in non-elite individuals (Blomstrand *et al.*, 1991). In laboratory controlled experiments, the ingestion of BCAA seems equivocal, with some reporting 12 % improvement in endurance capacity in warm environments (Mittleman *et al.*, 1998) while others have showed no such benefits (Cheuvront *et al.*, 2004; Watson *et al.*, 2004). Furthermore, the intakes of BCAA under thermoneutral environments seem to mimic the findings under heat stress (Madsen *et al.*, 1996; van Hall *et al.*, 1995). It should be noted, however, the lack of benefit may be linked to ammonia production that may negatively impact brain neurotransmitter metabolism (Meeusen, 2014). The co-ingestion of carbohydrate may also have a positive influence by blunting NEFA concentration and thus preventing the uptake of tryptophan (Blomstrand *et al.*, 2005) and/or via mechanisms related to stimulating reward centres in the brain and corticomotor excitability (for a review see (Jeukendrup, 2013)). At present, no positive effects to the addition of carbohydrate to BCAA have been reported (Cheuvront *et al.*, 2004; Madsen *et al.*, 1996).

To summarise, there is convincing evidence that fatigue not only resides in the periphery but central efferent and afferent signals appear to be involved with the onset of this phenomenon. Thus, targeting a single neurotransmitter may be overly reductionist as fatigue appears to be multifaceted and may occur at different locations simultaneously. Many peripheral factors such as hypoglycaemia and increased NEFA concentration are associated with CNS fatigue by altering brain monoamine levels or depleting the energy reservoir residing in this organ. Accordingly, it must be recognised that running to volitional exhaustion carries a CNS component.

## 2.3 Post-exercise Recovery

A number of factors encompass recovery including rehydration, regeneration and repair of damaged tissue and restoration of depleted carbohydrate stores (Betts and Williams, 2010). The restoration of endogenous carbohydrate stores is proposed to be crucial in determining the time required for recovery (Jentjens and Jeukendrup, 2003). In contrast to the predominant reliance on carbohydrate metabolism during prolonged exercise, post-exercise recovery is characterised by an accelerated rate of lipid oxidation ( $\approx 60\%$  of oxidative metabolism) and “sparing” of carbohydrate oxidation, even under conditions of high-carbohydrate feedings (Horton *et al.*, 1998; Kiens and Richter, 1998). This shift in substrate metabolism clearly demonstrates the high metabolic priority for muscle glycogen resynthesis, whereby lipid oxidation from intra and extra-muscular sources is elevated to meet the fuel requirements (Egan and Zierath, 2013). The process of glycogen resynthesis begins immediately following exercise and is most rapid during the first 5–6 h of recovery (Goforth *et al.*, 2003). Therefore, a view of glycogen resynthesis, the factors that enhance or limit muscle glycogen resynthesis and the nutritional strategies to obtain rapid post-exercise glycogen restoration are discussed.

### 2.3.1 Glucose transport

#### 2.3.1.1 Contraction-induced glucose uptake

Glucose uptake by the skeletal muscle occurs primarily by facilitated diffusion, which is mediated by the presence of glucose transporter 4 (GLUT4) from the intracellular storage vesicle to the cell surface (MacLean *et al.*, 2000; Stanford and Goodyear, 2014). The translocation of GLUT4 to the plasma membrane was shown to be modulated by exercise-induced muscle contraction and insulin through different molecular mechanisms (Brozinick *et al.*, 1994; Goodyear *et al.*, 1990b; Lund *et al.*, 1995; Thorell *et al.*, 1999), and both mechanisms have additive effects on muscle glucose uptake in humans (DeFronzo *et al.*, 1981; Nuutila *et al.*, 2000; Thorell *et al.*, 1999). Therefore, the presence of two distinct pools of glucose transporters has been proposed (Goodyear and Kahn, 1998), which was further substantiated by the observation that GLUT4 translocation becomes normal in type 2 diabetic patients following an acute exercise bout (Kennedy *et al.*, 1999). While there is substantial



evidence using different labelling and imaging techniques of tagged GLUT4 demonstrating that muscle contraction and insulin translocate this transporter protein to the cell surface (Karlsson *et al.*, 2009; Lauritzen *et al.*, 2010; Lauritzen *et al.*, 2006; Marette *et al.*, 1992), it remains a possibility that the intrinsic activity of GLUT4 may also be increased by stimuli such as insulin and exercise (Michelle Furtado *et al.*, 2003). However, it should be recognised that some methodological issues arise with the latter contention, as no direct assay of GLUT4 intrinsic activity is presently available and any changes regarding intrinsic activity must be inferred from accurate measurements of cell surface GLUT4 and glucose uptake in the same system (Richter and Hargreaves, 2013). Notwithstanding this possibility, the increases in glucose uptake can be quantitatively matched by the increased number of GLUT4 transport proteins and the maximal rate of glucose transport is determined by the number and proportion of GLUT4 translocated to the cell membrane in response to muscle contraction and/or insulin stimulation (Lund *et al.*, 1995; MacLean *et al.*, 2000). Thus, the consensus view at present is that glucose transport is primarily mediated by GLUT4 translocation (Lauritzen, 2009; Richter and Hargreaves, 2013).

There are a number of mechanisms related to the increased rate of glucose uptake via muscle contraction. The increase in blood flow during exercise may be considered as the primary contributor to muscle glucose uptake, as changes in plasma glucose concentration translate almost directly into proportional changes in leg glucose uptake (Rose and Richter, 2005). An increased cytoplasmic concentration of  $\text{Ca}^{2+}$  has been reported to increase glucose transport during exercise (Holloszy and Narahara, 1967, 1965), and the direct effects of  $\text{Ca}^{2+}$  were further supported by findings that glucose uptake can be stimulated by levels of  $\text{Ca}^{2+}$  release that were insufficient to induce muscular contractions (Youn *et al.*, 1991). It is noteworthy that caffeine was incubated to induce  $\text{Ca}^{2+}$  release from the sarcoplasmic reticulum in the aforementioned studies, which has also been shown to increase adenosine monophosphate-activated protein kinase (AMPK) activation in the absence of muscle contraction (Egawa *et al.*, 2011; Jensen *et al.*, 2007), presumably associated with the high energetic demands of  $\text{Ca}^{2+}$  reuptake by the sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) activity (Smith *et al.*, 2013). Indeed, the caffeine-mediated increase in glucose uptake was markedly impaired when caffeine was administered in mice with blunted endogenous AMPK activity (Jensen *et al.*, 2007). Thus, cytosolic  $\text{Ca}^{2+}$  release

may be related to an increased energy stress of the muscle secondary to caffeine-mediated activation of AMPK and the subsequent increased glucose uptake (Richter and Hargreaves, 2013).

Yet, the activation of other  $\text{Ca}^{2+}$  sensitive downstream signalling molecules may be implicated in contraction-induced glucose uptake such as calcium/calmodulin-dependent protein kinase (CaMK) and protein kinase C (PKC). An impaired muscle contraction-induced glucose uptake has been reported when CaMK or PKC were inhibited (Ihlemann *et al.*, 1999; Witczak *et al.*, 2010). However, contraction-induced glucose uptake was not impaired in PKC $\alpha$  ( $\approx 97$  % of total PKC) knockout mice (Jensen *et al.*, 2009). Nonetheless, it was recently demonstrated that sustained cytosolic  $\text{Ca}^{2+}$  by cyclopiazonic acid (a sarcoplasmic reticulum  $\text{Ca}^{2+}$  reuptake inhibitor) was insufficient to stimulate glucose transport and that the full contraction-induced glucose transport can be obtained with AMPK stimulation and mechanical stress by passive stretching without increased sarcoplasmic reticulum  $\text{Ca}^{2+}$  dependent signalling (Jensen *et al.*, 2014). Taken together, recent evidence suggests that increased AMPK activity and mechanical stress regulate glucose uptake during muscular contraction and that  $\text{Ca}^{2+}$  *per se* is unlikely to increase muscle glucose uptake. Rather, the known role of  $\text{Ca}^{2+}$  in causing muscle contraction and the ATP consumption by SERCAs instigates metabolic stress leading to the activation of AMPK and an increase in glucose uptake (Jensen *et al.*, 2014; Richter and Hargreaves, 2013; Smith *et al.*, 2013).

As depicted in Figure 2.3, there are many factors that can influence the rate of glucose uptake by the contracting skeletal muscle. Indeed, increasing pre-exercise glycogen content has been associated with a reduction in muscle glucose uptake, presumably via greater glycogenolysis and the resultant increase in glucose-6-phosphate and subsequent inhibition of hexokinase (Hespeel and Richter, 1990; Richter and Hargreaves, 2013). However, a reduction in muscle glycogen concentration has also been shown to increase glucose uptake, as evidenced by the inverse correlation ( $r = -0.53$ ) between GLUT4 translocation and muscle glycogen levels (Derave *et al.*, 1999). Although this may infer that glycogen may be structurally attached to GLUT4 (Richter *et al.*, 2001), there is no direct evidence to support this notion. In accordance, it is difficult to establish the direct link between muscle glycogen content and glucose

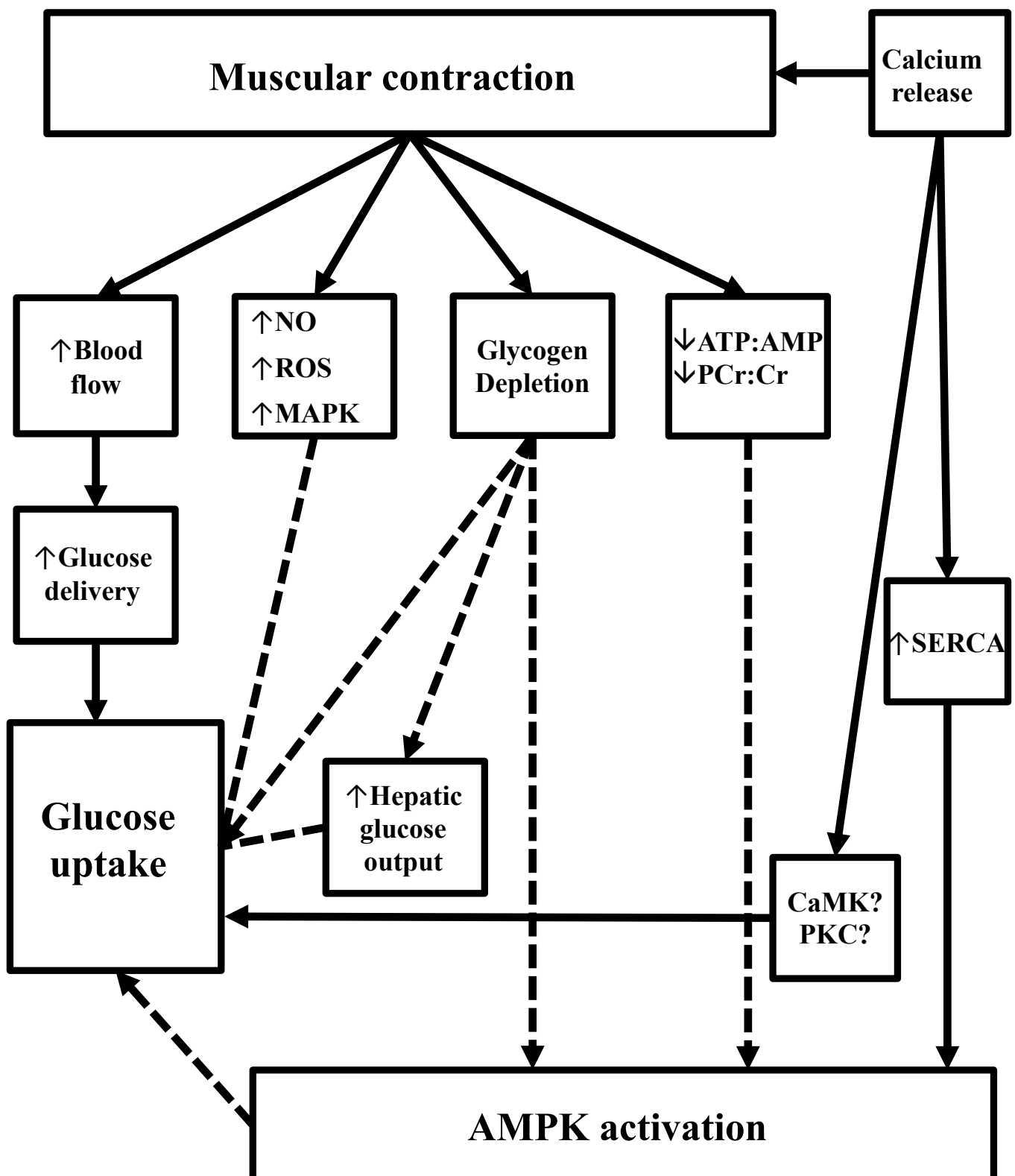
uptake (i.e. inhibition of glucose phosphorylation or glycogen-mediated translocation of GLUT4 to the cell membrane), mainly ascribed to confounding variables to the outcome measures such as dietary manipulation and hormonal levels (Steensberg *et al.*, 2002; Wojtaszewski *et al.*, 2003). Nevertheless, even when the delivery of substrates and hormones remains constant, glycogen content is inversely related to glucose uptake (Steensberg *et al.*, 2002). It should be recognised, however, that the reduction in muscle glycogen content is known to activate AMPK and increase glucose uptake (Wojtaszewski *et al.*, 2003), which is a mutual pathway to a number of other feedback mechanisms associated with an increase in glucose uptake. When considering the culmination of evidence, contraction-induced muscle glucose uptake can be mainly ascribed to AMPK activation (Richter and Hargreaves, 2013), albeit other mechanism may also be responsible (Figure 2.3).

### **2.3.1.2 Insulin-induced glucose uptake**

As alluded to in the previous section, there are distinct proximal signalling mechanisms responsible for GLUT4 translocation and glucose uptake by the muscle in response to exercise and insulin. This notion was further supported by findings that muscle-specific insulin receptor knockout mice showed normal contraction-induced glucose transport (Wojtaszewski *et al.*, 1999). In the absence of insulin  $\approx 90\%$  of GLUT4 remains within its intracellular storage vesicle (Thong *et al.*, 2005). Upon its stimulation, insulin causes a downstream cascade of molecular signalling events that are initiated by the phosphorylation of the insulin receptor substrate (IRS) on tyrosine residues (Goodyear *et al.*, 1995). This promotes IRS engagement with phosphatidylinositol 3-kinase (PI3-K) to generate phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>), facilitating the activation of Akt (also referred to as PKB) and consequently the translocation of GLUT4 to the cell surface (Cartee, 2014; Thong *et al.*, 2005). Emerging evidence also indicates converging signalling pathways for insulin and exercise-induced glucose uptake, namely the phosphorylation of tre-2/USP6, BUB2, cdc16 domain family member 1 (TBC1D1) and Akt substrate of 160 kDa (AS160) to stimulate glucose GLUT4 transport (Stanford and Goodyear, 2014). Although the current understanding regarding these “converging” pathways is far from comprehensive, this may help in explaining why acute exercise improves insulin sensitivity by increasing the phosphorylation of downstream signalling proteins (e.g.

Akt) in the cytosolic fraction without altering the proximal insulin signalling pathways such as IRS and PI3-K (Wilson *et al.*, 2006).

It is clear from the above that insulin-stimulated glucose uptake is a complex phenomenon that involves a number of regulatory steps. Nevertheless, it is established that increasing glucose and insulin concentrations in the acute post-exercise recovery period mediates large increases in glucose transport (Kuo *et al.*, 1999; Kuo *et al.*, 2004). This is primarily ascribed to an accelerated insulin-stimulated glucose transport (i.e. insulin sensitivity) secondary to GLUT4 translocation to the cell surface following an acute exercise bout (Cartee and Funai, 2009; Cartee *et al.*, 1989). The magnitude of glycogen depletion may therefore be a candidate in the enhanced sensitivity to insulin, as demonstrated by the inverse correlation ( $r = -0.67$ ;  $p < 0.05$ ) between muscle glycogen content and insulin-stimulated glucose uptake (Jensen *et al.*, 1997). Furthermore, GLUT4 protein content on the cell surface was negatively correlated ( $r = -0.53$ ;  $p < 0.05$ ) with glycogen content during insulin stimulation (Derave *et al.*, 1999), indicating that insulin-mediated GLUT4 translocation is regulated by muscle glycogen content. Interestingly, the augmented insulin sensitivity following exercise was shown to persist for 48-h in a carbohydrate-deprived state, and the reversal of this effect occurs with carbohydrate feeding and concomitant with glycogen supercompensation (Cartee *et al.*, 1989).



**Figure 2.3** Regulation of glucose uptake during/after exercise. Solid arrows represent feed-forward mechanisms and dashed arrows represent feed-back mechanisms.

Notably, low muscle glycogen levels were associated with an enhanced Akt phosphorylation and activity without changing the early steps of proximal insulin signalling, suggesting that increased Akt activity may contribute to the enhanced sensitivity to insulin in muscle with low glycogen (Jensen *et al.*, 2006; Lai *et al.*, 2010). Notwithstanding these direct links between muscle glycogen and insulin induced glucose uptake, some evidence also supports the notion that insulin sensitivity may be mediated by AMPK activation, presumably due the potential AMPK binding to glycogen through  $\beta$  subunits (Fisher *et al.*, 2002; Friedrichsen *et al.*, 2013; McBride *et al.*, 2009). In accordance, glycogen depletion may also exert an indirect effect of insulin sensitivity via AMPK activation. It is worth noting that exercise involving eccentric contraction which induces muscle damage may impair insulin sensitivity in the days following exercise, mainly ascribed to decreased GLUT4 expression and impaired insulin signalling (Maarbjerg *et al.*, 2011).

### **2.3.2 Skeletal muscle glycogen**

Rapid muscle glycogen repletion requires high rates of glucose to be taken up by the skeletal muscle, the major tissue for total body glucose disposal (DeFronzo *et al.*, 1981). Hence, the most potent and physiologically relevant stimuli of glucose transport into the skeletal muscle (contractile activity and insulin sensitivity) and their distinct mechanisms to regulate glucose uptake have been considered in the previous section. However, to be stored within the muscle as fuel storage for exercise, glucose entering the muscle must be converted to glycogen.

#### **2.3.2.1 Glycogen structure and localisation**

Skeletal muscle glycogen is a highly optimised efficient cellular energy storage system whereby its branched structure allows expeditious availability of large amounts of glucose to support the energetic demands of muscular contractions (Melendez *et al.*, 1999). The biosynthesis of this granule involves three enzymes; glycogenin, glycogen synthase (GS) and branching enzyme (Alonso *et al.*, 1995). The primer in glycogen synthesis is the protein glycogenin, which incorporates glucose residues through a self-glucosylation reaction and then acts as a substrate for GS and branching enzyme to form two physiologically distinct glycogen pools (Graham *et al.*,

2010). Namely, proglycogen (low molecular weight acid-insoluble glycogen) and macroglycogen (high molecular weight acid-soluble glycogen) are formed from the core molecule glycogenin, with the latter being an expansion of the first (Alonso *et al.*, 1995). These two distinct pools were shown to differ in their rates of degradation and synthesis, in addition to their sensitivity to dietary manipulation (Adamo and Graham, 1998; Adamo *et al.*, 1998; Graham *et al.*, 2001). Accordingly, proglycogen storage was reported to be the most prominent during the initial post-exercise phase due to its greater sensitivity to the provision of dietary carbohydrate and a more rapid glycogen synthesis rate following glycogen depletion (Adamo *et al.*, 1998). At the other end of the glycogen continuum, macroglycogen was shown to be restored in a constant slower rate and can persist for 2 – 3 days (Adamo *et al.*, 1998). Nevertheless, while it is known that muscle glycogen can be separated into distinct acid-soluble and acid-insoluble fractions (Jansson, 1981), the acid-insoluble glycogen does not correspond to proglycogen as both the acid-soluble and acid-insoluble glycogen show similar elution profiles of high molecular weight glycogen using gel chromatography (James *et al.*, 2008). The latter finding certainly questions the existence of proglycogen (i.e. low-molecular weight glycogen) as a distinct pool of glycogen and thus the proglycogen-macroglycogen paradigm may be an artefact of glycogen analysis.

Apart from its structure, the location of glycogen appears to be an important factor. The majority of investigations utilised acid-base digestion and subsequent enzymatic determination of free glucose for quantification of total muscle glycogen. While this method provides valuable knowledge on glycogen-mediated whole muscle metabolism, this does not allow for examination of glycogen localisation. More recently, transmission electron microscopy (TEM) has been utilised to determine the subcellular localisation of glycogen within the fibres. This approach has led to the appreciation that glycogen is primarily distributed in subsarcolemmal, inter-myofibrillar and intra-myofibrillar pools (Nielsen *et al.*, 2011; Ortenblad *et al.*, 2013) and that resynthesis of glycogen following exercise was characterised initially by an increase in granule number and later by an increase in size (Marchand *et al.*, 2007). This glycogen heterogeneity likely provides a substrate for specific cellular functions, which is supported when considering the preferential depletion of intra-myofibrillar glycogen during prolonged exercise and the relative distribution of these distinct

pools being largely dependent on fibre type, training status, immobilisation and exercise (Nielsen and Ortenblad, 2013).

### 2.3.2.2 Glycogen resynthesis

Upon entering the muscle cell, glucose is rapidly converted to G6P by hexokinase, which is then converted to glucose-1-phosphate (G1P) via phosphoglucomutase. Thereafter, G1P and uridine triphosphate interact to generate uridine diphosphate glucose (UDP-glucose), which serves as a glycosyl carrier (Ivy, 1991). The glucose residue is subsequently linked to a pre-existing glycogen chain (amylose chain) by an  $\alpha$ -1,4 glycosidic bond, catalysed by the enzyme glycogen synthase (GS) (Ivy and Kuo, 1998; Jentjens and Jeukendrup, 2003). When the elongating glycogen chain consists of at least 11 glucose residues, branching enzyme transfers a fragment of 6-7 glucose residues to nearby chain by an  $\alpha$ -1,6 bond (Greenberg *et al.*, 2006). Cumulatively, these cellular processes illustrate that GS elongates the glycogen chain, while branching enzyme produces new branches that create a compact spherical glycogen particle (Melendez *et al.*, 1999).

In concert with glucose transport into the muscle, GS activity is thought to be a rate-limiting step in glycogen resynthesis (Ivy, 1991; Ivy and Kuo, 1998). The activity of this enzyme is controlled by reversible phosphorylation-dephosphorylation leading to GS inactivation and activation, respectively (Danforth, 1965; Nielsen and Richter, 2003). GS is regulated by a complex sequence of hierarchical phosphorylations involving several protein kinases such as CaMKII, PKC and AMPK (Roach, 2002), although their relative importance on the regulation of GS *in vivo* is not fully understood (Prats *et al.*, 2009). Another mechanism by which GS is regulated is via its binding G6P, resulting in allosteric activation that overrides inhibition by phosphorylation and promotes dephosphorylation of the enzyme (Greenberg *et al.*, 2006). The translocation of GS from the glycogen granule to the membrane and cytosolic fraction can be considered as a third mechanism by which the enzyme is regulated (Nielsen *et al.*, 2001; Prats *et al.*, 2005; Prats *et al.*, 2009).



Consistent with other factors relating to glycogen resynthesis, muscular contraction, low glycogen content and insulin are regulators of GS activity. There is compelling evidence that GS activity and muscle glycogen content exhibit a tightly controlled inverse relationship (Bergstrom *et al.*, 1972; Danforth, 1965; Jensen *et al.*, 2006; Zachwieja *et al.*, 1991). Further support of this notion is the observation that patients with McArdle's disease demonstrate no activation of GS following exercise when compared with a control group (Nielsen and Richter, 2003). A number of physiological mechanisms may act in concert (GS-protein interactions) to contribute to the aforementioned relationship such as protein phosphatase I, malin, laforin and AMPK, but the regulation of these remains far from understood (Graham *et al.*, 2010; Nielsen and Richter, 2003). Notwithstanding the complexities associated with GS activation, glycogen content and muscular contraction *per se* have been implicated to independently regulate GS affinity to UDP-glucose (Lai *et al.*, 2007).

Additionally, there is substantial evidence demonstrating that insulin positively influences GS activity, which is primarily associated with an increased sensitivity to G6P (Danforth, 1965; Lawrence and Roach, 1997). The initial steps in the signalling pathways for insulin-stimulated activation of GS are similar to those described to translocate GLUT4, such that phosphorylation of IRS and the downstream activation of Akt are present (Jensen *et al.*, 2006). While the molecular link between Akt and glucose uptake is not fully established, it appears that Akt directly phosphorylates and deactivates the inhibitory glycogen synthase kinase (GSK)-3 and thus stimulating GS activity (Cross *et al.*, 1995). Interestingly, muscular contraction and insulin were shown to additively increase GS activity with normal and high glycogen content but not when glycogen levels were low. This was despite the fact the GS activity was greatest in the low glycogen trial, irrespective of muscle contraction or insulin stimulation (Lai *et al.*, 2010). These findings clearly demonstrate that while GS activity is regulated by muscular contraction and insulin stimuli, muscle glycogen content can be considered as the most potent regulator of GS activity, at least in the acute stages during post-exercise recovery.

### 2.3.2.3 The two phases of muscle glycogen resynthesis

It has been indicated that glycogen resynthesis after a bout of exercise occurs in a biphasic pattern (Maehlum *et al.*, 1977; Price *et al.*, 1996; Price *et al.*, 1994). Initially, there is a rapid increase in glycogen resynthesis at a rate of  $\approx 45 \text{ mmol} \cdot \text{kg} \cdot \text{dm}^{-1} \cdot \text{h}^{-1}$ , which occurs independent of insulin concentrations and lasts for 30-60 minutes post-exercise (Ivy and Kuo, 1998; Jentjens and Jeukendrup, 2003). The presence of this phase is supported by data reporting an accelerated rate of muscle glycogen resynthesis in the initial (0-60 min) post-exercise period, even when insulin was inhibited by somatostatin or when insulin resistant individuals were compared to healthy age-matched controls (Price *et al.*, 1996; Price *et al.*, 1994). However, this rate of resynthesis can rapidly decline in the absence of exogenous carbohydrate intake (Ivy *et al.*, 1988a; Maehlum *et al.*, 1977). These findings are in agreement with the time course of the protracted increase in glucose uptake after exercise, whereby a twofold increase in GLUT4 translocation can be observed immediately following exercise before gradually declining until reaching pre-exercise levels by 2 h upon cessation (Goodyear *et al.*, 1990a). This insulin-independent phase was suggested to only occur when glycogen is depleted to critically low levels ( $\approx 150 \text{ mmol} \cdot \text{kg} \cdot \text{dm}^{-1} \cdot \text{h}^{-1}$ ) at the end of an exercise bout (Jentjens and Jeukendrup, 2003; Maehlum *et al.*, 1977). Therefore, it appears that the mechanisms responsible for this initial and rapid phase of glycogen restoration involve exercise-induced GLUT4 translocation to the cell membrane and an augmented GS activity secondary to low glycogen concentrations at the end of exercise (Ivy and Kuo, 1998; Jentjens and Jeukendrup, 2003).

The second phase of glycogen storage is thought to occur at a substantially lower rate (approximately 80 % lower), and is characterised by the affinity of muscle glucose uptake and GS to insulin stimulation (Cartee *et al.*, 1989; Danforth, 1965). Again this is supported when examining insulin resistant individuals who only show 3.5 % ability to resynthesise muscle glycogen ( $0.4 \text{ mmol} \cdot \text{kg} \cdot \text{dm}^{-1} \cdot \text{h}^{-1}$ ) relative to healthy controls (Price *et al.*, 1996). The ingestion of carbohydrate and the associated increase in glucose and insulin concentrations are known to accelerate the rate of muscle glycogen resynthesis during this phase, albeit they remain slower  $\approx 30 \text{ mmol} \cdot \text{kg} \cdot \text{dm}^{-1} \cdot \text{h}^{-1}$  than the insulin-independent phase (Ivy *et al.*, 1988a; Ivy *et al.*, 1988b; Keizer *et*

*al.*, 1987). This greater muscle sensitivity to insulin can persist for longer periods (>48 h) and is reliant on carbohydrate ingestion and the amount of muscle glycogen that has been restored (Cartee *et al.*, 1989; Lai *et al.*, 2010). As mentioned in section 2.3.2.2, a number of factors related to the enhanced muscle insulin sensitivity, such as insulin-mediated GLUT4 translocation, increased sensitivity of GS to insulin, muscle glycogen content and AMPK activation (Fisher *et al.*, 2002; Ivy and Kuo, 1998; Wojtaszewski *et al.*, 2003).

## 2.4 Nutrient intake and muscle glycogen resynthesis

Glucose is the precursor for glycogen resynthesis and consequently the amount carbohydrate ingested can be considered as the most important factor determining the restoration of glycogen (Beelen *et al.*, 2010; Burke *et al.*, 2004; Jentjens and Jeukendrup, 2003). It has been consistently demonstrated that carbohydrate intake increases glycogen storage above that of water alone (Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997; van Hall *et al.*, 2000). In the context of recovery from exhaustive exercise, it is known that ingesting 6-12 g carbohydrate·kg<sup>-1</sup> is sufficient to restore the body's glycogen reserves when the recovery time is ≥24 h (Burke *et al.*, 2011; Jensen *et al.*, 2015). However, athletes and active individuals across a wide range of sporting events train and compete at levels that challenge their daily glycogen stores with minimal time for recovery, with multiple training sessions a day and/or a daily competitive schedule with a fuel cost that exceeds the endogenous carbohydrate stores. Thus, when the time available for recovery is limited (< 8 h), neither muscle glycogen nor the capacity for subsequent exercise can be fully restored (Betts and Williams, 2010). It becomes apparent that specific nutritional strategies aimed at acutely maximising glycogen resynthesis are an important consideration in such scenarios. A related but separate question is whether the adaptive response to chronic training is amplified when commencing a number of exercise sessions with low-muscle glycogen concentrations. A review on the role of endogenous carbohydrate availability on endurance training adaptations will be briefly discussed in section 2.6.2 and the reader is referred to a more extensive report on this topic elsewhere (Bartlett *et al.*, 2015; Philp *et al.*, 2012).

Attempts to identify the optimal carbohydrate feeding strategy to maximise glycogen reserves vary greatly and depend on a number of factors that include but are not limited to the amount, timing and type of the carbohydrate ingested during recovery (Betts and Williams, 2010; Jentjens and Jeukendrup, 2003). Additionally, there is some evidence that including other macronutrients, namely protein, may aid in augmenting muscle glycogen synthetic response (Berardi *et al.*, 2006; Zawadzki *et al.*, 1992). Therefore, the following sections will discuss these nutritional considerations related to glycogen resynthesis during short-term recovery.

#### **2.4.1 Amount of carbohydrate intake**

In the absence of carbohydrate ingestion over the post-exercise recovery period, little glycogen resynthesis occurs at a rate of  $\approx 2 \text{ mmol} \cdot \text{kg} \text{ dm}^{-1} \cdot \text{h}^{-1}$  (Ivy *et al.*, 1988b; Maehlum and Hermansen, 1978). Coupled with the wealth of knowledge demonstrating that any ingested carbohydrate of substantial amounts greatly increases muscle glycogen resynthesis than when no carbohydrate is ingested (Ferguson-Stegall *et al.*, 2011b; Gejl *et al.*, 2014; Ivy *et al.*, 1988b; Jensen *et al.*, 2015; Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997; van Hall *et al.*, 2000) enforces the notion that carbohydrate ingestion is critical for restoration of muscle glycogen. The first study to explore the effects of varying amounts of carbohydrate on muscle glycogen resynthesis during short-term recovery showed that increasing carbohydrate ingestion from  $0.18 \text{ g} \cdot \text{kg} \text{ BM}^{-1} \cdot \text{h}^{-1}$  to  $0.35 \text{ g} \cdot \text{kg} \text{ BM}^{-1} \cdot \text{h}^{-1}$  concurrently enhances glycogen synthesis rate from 9 to  $25 \text{ mmol} \cdot \text{kg} \text{ dm}^{-1} \cdot \text{h}^{-1}$  (Blom *et al.*, 1987). When increasing the amount of carbohydrate to  $0.70 \text{ g} \cdot \text{kg} \text{ BM}^{-1} \cdot \text{h}^{-1}$ , the authors reported no further increase in glycogen resynthesis rate (Blom *et al.*, 1987). Indeed, these observations may suggest that the former rate of ingestion would maximise glycogen synthesis. However, a number of following studies demonstrated that increasing the rate of carbohydrate ingestion from  $0.75 - 1 \text{ g} \cdot \text{kg} \text{ BM}^{-1} \cdot \text{h}^{-1}$  elicits a greater glycogen synthetic response than reported previously (Blom, 1989; Casey *et al.*, 1995; Shearer *et al.*, 2005; Zachwieja *et al.*, 1991). It was elegantly demonstrated that carbohydrate ingestion at a rate of  $1.2 \text{ g} \cdot \text{kg} \text{ BM}^{-1} \cdot \text{h}^{-1}$  during post-exercise recovery resulted in 150 % greater glycogen synthetic response (from 17 to  $45 \text{ mmol} \cdot \text{kg} \text{ dm}^{-1} \cdot \text{h}^{-1}$ ) relative to a

lower dose of  $0.8 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  (van Loon *et al.*, 2000b). Because the ingestion of  $1.6 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  of carbohydrate does not further stimulate muscle glycogen resynthesis above that of  $1.2 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  (Howarth *et al.*, 2009) the latter may be considered as the optimal amount to maximise muscle glycogen repletion.

Identifying the precise ‘optimal’ quantity of carbohydrate to maximise glycogen repletion is difficult to ascertain, which may be ascribed to a number of confounding variables including the type and timing of the ingested carbohydrate, the training status of the participants and the duration of the post-exercise recovery period. More importantly, however, the magnitude of muscle glycogen depletion determines to a large extent its rate of resynthesis (Zachwieja *et al.*, 1991). It is therefore notable that the degree of glycogen depletion from a prior exercise bout varies substantially across the literature, such that a range of muscle glycogen concentrations ranging between  $25\text{--}255 \text{ mmol} \cdot \text{kg dm}^{-1} \cdot \text{h}^{-1}$  at the onset of recovery has been reported (Casey *et al.*, 1995; Tsintzas *et al.*, 2003). Together with the known inverse relationship between muscle glycogen content and glucose uptake (Derave *et al.*, 1999; Jensen *et al.*, 1997; Steensberg *et al.*, 2002), the variation in muscle glycogen levels at the onset of recovery are likely to contribute to the large variability in muscle glycogen resynthesis rates between studies. Nonetheless, a positive correlation ( $r = 0.6$ ;  $p < 0.01$ ) exists between the amount of carbohydrate ingested during short-term recovery and muscle glycogen resynthesis (Betts and Williams, 2010). Collectively, it is reasonable to suggest that ingesting  $\approx 1.2 \text{ g carbohydrate} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  is likely to maximise muscle glycogen resynthesis and that additional carbohydrate will not further increase this glycogenic response.

#### **2.4.2 Type of carbohydrate intake**

A number of studies explored different types of carbohydrate ingestion during short-term recovery in an attempt to establish the most effective means to maximise glycogen storage. An important factor determining the rate of muscle glycogen resynthesis is insulin-mediated glucose uptake by the muscle cells (Jentjens and Jeukendrup, 2003). As such, the elevated insulinaemic response to high as opposed to a low glycaemic index carbohydrate is implicated to accelerate muscle glycogen

repletion, at least in the acute (< 6 h) recovery phase, with no distinct advantage in longer recovery periods (Kiens *et al.*, 1990). In contrast, differences in muscle glycogen storage favouring high glycaemic index carbohydrate were shown to persist up to 24 h (Burke *et al.*, 1993). The authors of the latter postulated that these differences may be attributable to the malabsorption of carbohydrate in the low glycaemic index foods, which enforces the view that the type of carbohydrate ingestion is an important consideration in relation to muscle glycogen resynthesis. Of note, a study by Wee *et al.* (2005) demonstrated that a high-glycaemic index meal ingested 3 h prior to exercise increases muscle glycogen content more than when an isoenergetic low-glycaemic index meal was ingested (Wee *et al.*, 2005). While this pre-exercise meal may be extrapolated to reflect a recovery meal prior to exercise, the data from this study must be interpreted with caution, as the metabolic perturbations to a repeated exercise bout remain largely unknown and may differ from a prior exercise bout.

Fructose mediates modest amounts of insulin stimulation relative to glucose and sucrose, mainly ascribed to its preferential hepatic glycogen resynthesis (Delarue *et al.*, 1993). As a consequence, fructose ingestion does not appear to effectively stimulate muscle glycogen resynthesis when compared to glucose or sucrose (Bergstrom and Hultman, 1967b; Nilsson and Hultman, 1974), which is also supported by  $^{13}\text{C}$ -NMR data (Van Den Bergh *et al.*, 1996). Furthermore, it appears that sucrose and glucose stimulate muscle glycogen resynthesis at a similar magnitude (Blom *et al.*, 1987). More recently, it was demonstrated that when  $1.2 \text{ g carbohydrate} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  are ingested during recovery from an initial exhaustive bout, glucose and glucose + fructose ingestion elicit similar muscle glycogen resynthesis rates (Wallis *et al.*, 2008). It would therefore be prudent to ingest a mixture of glucose and fructose to provide an optimal dose of carbohydrate for the effective restoration of both muscle and liver glycogen stores (Blom *et al.*, 1987; Casey *et al.*, 2000; Wallis *et al.*, 2008). Owing to the fact that sucrose contains equimolar amounts of glucose and fructose infers that this disaccharide would be favourable to optimise overall endogenous carbohydrate reserves (liver and muscle glycogen), both of which have been shown to associate with the capacity for exercise (Casey *et al.*, 2000).

Manipulation of the form (solid *versus* liquid) of carbohydrate ingestion does not seem to influence the rate of muscle glycogen resynthesis during recovery (Blom, 1989; Keizer *et al.*, 1987; Reed *et al.*, 1989). These observations are in concert of the view that muscle glycogen resynthesis is unlikely to be limited by gastric emptying (Reed *et al.*, 1989). Rather, a combination of other factors such as the amount of carbohydrate, intestinal absorption and delivery into circulation, extraction of glucose by other tissues and the capacity for glucose transport into the muscle (Jentjens and Jeukendrup, 2003). In relation to this contention, it is interesting to note that the intestinal absorption of fructose occurs through a different transporter (sodium-independent glucose transporter protein 5; GLUT-5) than glucose (sodium-dependent glucose transporter 1; SGLT1) and thus the combined ingestion of glucose and fructose may accelerate overall carbohydrate delivery, which is an important consideration during acute post-exercise recovery, particularly when large amounts ( $\geq 60 \text{ g}\cdot\text{h}^{-1}$ ) of glucose are ingested and thus saturating SGLT1 (Lecoultre *et al.*, 2010; Wallis and Wittekind, 2013).

#### **2.4.3 Timing of carbohydrate intake**

Skeletal muscle sensitivity to nutrient provision is thought to be augmented following a prior exercise bout that substantially depletes muscle glycogen, which emphasises the influence of timing of carbohydrate provision during the post-exercise recovery (Ivy, 2014). In fact, the rate of muscle glycogen resynthesis was shown to be  $\approx 25 \text{ mmol}\cdot\text{kg dm}^{-1}\cdot\text{h}^{-1}$  over 4 h of recovery when carbohydrate was provided immediately following exercise (Ivy *et al.*, 1988a). When carbohydrate provision was delayed by only 2 h, a considerable reduction to  $14 \text{ mmol}\cdot\text{kg dm}^{-1}\cdot\text{h}^{-1}$  in the rate of muscle glycogen resynthesis was reported (Ivy *et al.*, 1988a). In concurrence, delaying the ingestion of a mixed macronutrient recovery beverage by 3 h was shown to reduce net leg glucose uptake by 65 % during recovery when compared to immediate ingestion (Levenhagen *et al.*, 2001). These findings may arguably be expected given that insulin sensitivity and the capacity for glucose uptake are most rapid in the initial few hours of recovery, and when carbohydrate is withheld during this period rapid reversal of these effects can be observed (Cartee *et al.*, 1989; Goodyear *et al.*, 1990a; Ivy, 2001).

Nevertheless, a study by Parkin and colleagues (1997) sought to determine the effects of delayed carbohydrate feeding of 2 h on the recovery of muscle glycogen over 8 h of recovery and reported no differences in muscle glycogen resynthesis rates (Parkin *et al.*, 1997). While these findings may appear contradictory, it remains possible that glycogen resynthesis rates were higher in the study of Parkin *et al.* (1997) over the initial 4 h of recovery, with the reversal of this augmented glycogen synthetic rate later in recovery secondary to the well-established and tightly controlled inverse relationship between muscle glycogen content and GS activity (Jensen *et al.*, 2006). Therefore, it would not be unreasonable to postulate that the effect of timing of carbohydrate on muscle glycogen resynthesis is magnified when recovery time is shorter. Irrespective of the progressive insulin resistance that occurs later in recovery, it would be logical consume carbohydrate as soon as practically possible to initiate the effective time for muscle glycogen resynthesis.

Similar to the effects of the glycaemic index of foods during longer recovery periods (i.e. 24 h), the frequency of carbohydrate intake does not appear to influence overall muscle glycogen resynthesis (Burke *et al.*, 1996; Costill *et al.*, 1981). However, when recovery time is limited, the frequency at which the carbohydrate is ingested may have an influence. Specifically, those studies that adopted a feeding regimen at 2 h intervals typically reported muscle glycogen resynthesis rates between 14-25 mmol·kg<sup>-1</sup>·h<sup>-1</sup> (Blom *et al.*, 1987; Ivy *et al.*, 1988a; Ivy *et al.*, 1988b; Reed *et al.*, 1989). It may therefore be argued that the aforementioned frequency may not be a sufficient nutritional strategy to maintain the elevation in insulin and consequently maximise the activation of GLUT-4 and GS (Ivy, 1998). On the other hand, when carbohydrate feeding occurs within 15-30 minute intervals, muscle glycogen resynthesis rate has been found to be approximately 40 % higher than when supplementing every 2 h (Doyle *et al.*, 1993; Jentjens *et al.*, 2001; van Hall *et al.*, 2000; van Loon *et al.*, 2000b; Wallis *et al.*, 2008). It should be recognised, however, that there is currently no study that directly examined the frequency of supplementation on the rate of muscle glycogen storage. Nonetheless, it is reasonable to suggest from the studies cited above that when a more rapid glycogen restoration is required during short-term recovery, a more frequent feeding pattern may be favourable to achieve this target.



#### **2.4.4 Protein co-ingestion with carbohydrate**

##### **2.4.4.1 Protein co-ingestion and insulin secretion**

While it is known that glucose is the major stimulus for pancreatic insulin secretion, the observation that insulin concentrations are significantly stimulated when healthy individuals ingest protein or receive a mixture of amino acids intravenously provides a scientific basis for the role of amino acids in insulin stimulation (Floyd *et al.*, 1966a, 1966b). Moreover, amino acids act synergistically when co-ingested with carbohydrate to potentiate insulin secretion (Floyd *et al.*, 1970; Rabinowitz *et al.*, 1966; van Loon *et al.*, 2000a). The oral ingestion of an amino acid mixture, particularly with sufficient amounts of leucine and phenylalanine, produce strong insulintropic properties compared with carbohydrate only solutions (van Loon *et al.*, 2000a). It was also demonstrated that a hydrolysed protein fraction provides a distinct advantage in stimulating insulin release over its intact form, mainly related to an accelerated rate of digestion and absorption and the resultant increased plasma amino acid availability of the former (Koopman *et al.*, 2009; Morifuji *et al.*, 2010a). Furthermore, whey protein is a greater insulin secretagogue than casein (Reitelseder *et al.* 2011), presumably associated with the greater leucine content combined with its rapid plasma amino acid availability.

In addition to the findings regarding the most effective amino acid/protein fraction cited above, a further point related to the amount of protein to effectively stimulate insulin warrants discussion. It was previously suggested that increasing the amount of protein in a carbohydrate-protein mixture from 0.2 to 0.4 g·kg BM<sup>-1</sup>·h<sup>-1</sup> may be associated with greater insulin response, albeit these differences were only significant when reported as an incremental area under the curve (van Loon *et al.*, 2000c). A possible explanation would be the study provided wheat protein as opposed to whey protein, which contains substantially higher leucine content than the former. These differences may have considerable implications as leucine is a known modulator of insulin signalling pathway (Di Camillo *et al.*, 2014). This was supported by the same study whereby free leucine and phenylalanine were added to the wheat protein fraction and consequently increased insulin concentration relative to carbohydrate-

only beverage (van Loon *et al.*, 2000c). More recently, a dose-dependent relationship between the amount of whey protein co-ingested during recovery and insulin secretion was found when 0.1 compared to 0.3 g·kg BM<sup>-1</sup>·h<sup>-1</sup> were ingested (Morifuji *et al.*, 2012). When considered collectively, the available evidence that reported insulinotropic properties with carbohydrate-protein ingestion have included 0.3-0.4 g·kg BM<sup>-1</sup>·h<sup>-1</sup> of protein (Beelen *et al.*, 2012; Betts *et al.*, 2008; Jentjens *et al.*, 2001; van Hall *et al.*, 2000; Zawadzki *et al.*, 1992). In contrast, protein was provided at a lower dose in those studies that showed no effect of protein co-ingestion on insulin secretion relative to carbohydrate-only (Carrithers *et al.*, 2000; Ivy *et al.*, 2002; Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997).

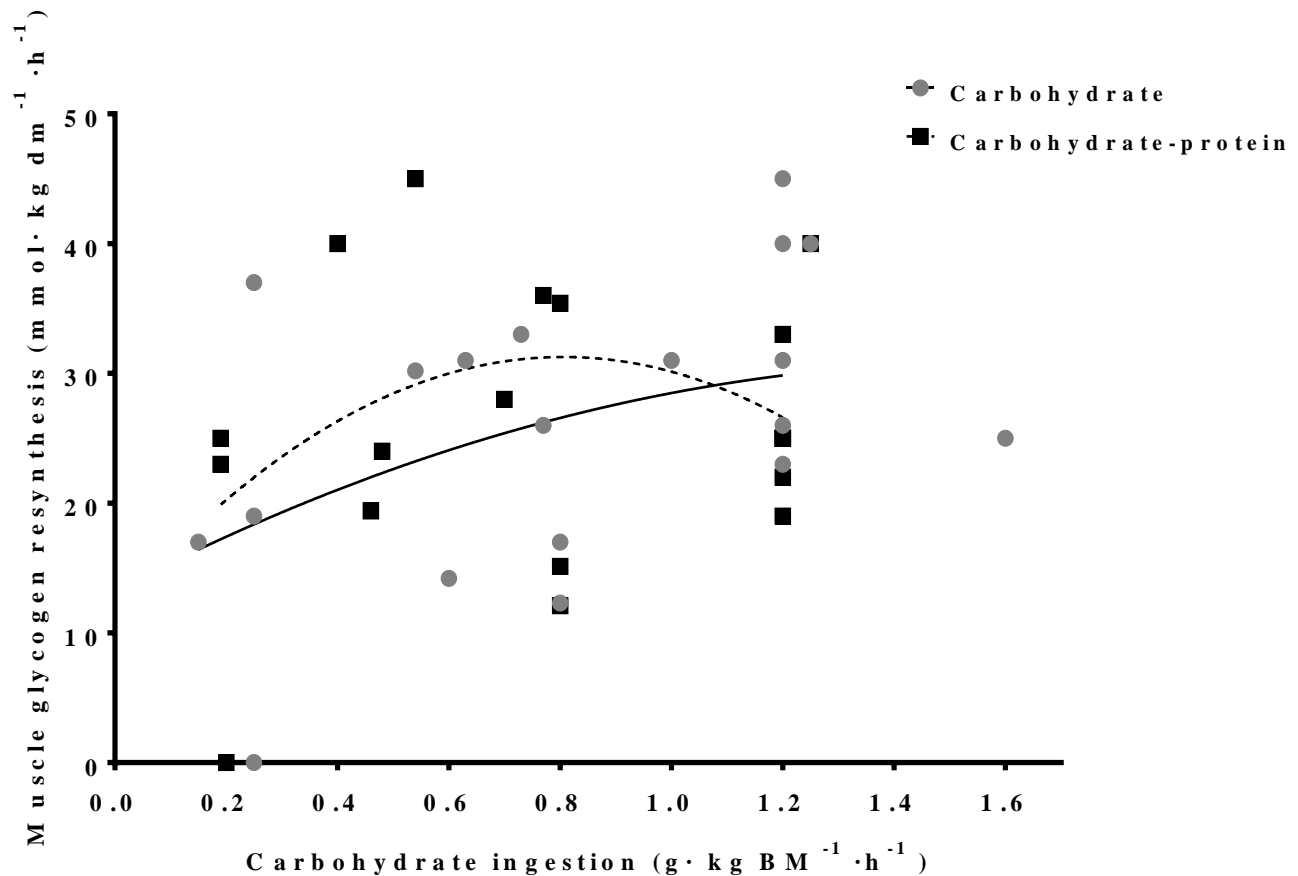
#### 2.4.4.2 Protein co-ingestion and glycogen resynthesis

As discussed in previous sections, the role of insulin in mediating glucose uptake and GS activity is a central rationale for the potential effect of protein co-ingestion with carbohydrate in accelerating muscle glycogen resynthesis. In this regard, the addition of whey protein hydrolysate (with or without additional free essential amino acids) to a carbohydrate supplement is known to result in a greater insulin response (Kaastra *et al.*, 2006; Reitelseder *et al.*, 2011; van Loon *et al.*, 2000a). Concurrently, adding protein to carbohydrate following recovery was reported to accelerate the rate of muscle glycogen resynthesis relative to a carbohydrate-only supplement when ingested in moderate amounts (i.e.  $\leq 0.8$  g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup>) (Berardi *et al.*, 2006; Hara *et al.*, 2011; Ivy *et al.*, 2002; Morifuji *et al.*, 2010b; van Loon *et al.*, 2000b; Williams *et al.*, 2003; Zawadzki *et al.*, 1992). Nevertheless, one of these studies did not match the carbohydrate content between the experimental beverages by providing only 38 % of the carbohydrate in the carbohydrate-protein trial, making it difficult to establish whether the glycogenic effects were due to the amount of carbohydrate ingested or the additional protein between supplements (Williams *et al.*, 2003).

While three of the above cited investigations matched the carbohydrate content between supplements, it is difficult to conclude whether the addition of protein or the higher caloric intake was related to the enhanced glycogen resynthesis rate (Hara *et al.*, 2011; Morifuji *et al.*, 2010b; Zawadzki *et al.*, 1992). In two investigations, however, muscle glycogen resynthesis was augmented when an isoenergetic carbohydrate-protein supplement was provided (Berardi *et al.*, 2006; Ivy *et al.*, 2002). Notably, muscle glycogen resynthesis was accelerated in the study of Ivy *et al.* (2002) irrespective of the fact that insulin concentrations during recovery were similar between supplements. Coupled with the fact that insulin concentrations were not reported in the other investigation (Berardi *et al.*, 2006), whether the enhanced glycogenic effect in those investigations was related to hyperinsulinaemia is questionable. The only remaining study is of particular interest owing to its comprehensive experimental design, as a carbohydrate-protein mixture (0.8 g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup> plus 0.4 g protein·kg BM<sup>-1</sup>·h<sup>-1</sup>) was examined against both a carbohydrate-matched (0.8 g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup>) and an energy-matched (1.2

g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup>) carbohydrate supplement (van Loon *et al.*, 2000b). The authors demonstrated that the addition of protein effectively increased insulin concentrations and glycogen storage by twofold when compared to the carbohydrate-matched supplement. However, replacing the protein fraction by additional energy in the form of carbohydrate achieved similar results, with no differences in glycogen resynthesis between the carbohydrate-protein mixture and the isoenergetic carbohydrate supplement (van Loon *et al.*, 2000b). The latter findings clearly demonstrate that carbohydrate intake should be greater than 0.8 g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup> to allow maximal glycogen resynthesis rates.

Equally, a number of other investigations proposed that the addition of protein does not further increase the rate of muscle glycogen resynthesis despite a higher insulinaemic response (Beelen *et al.*, 2012; Betts *et al.*, 2008; Detko *et al.*, 2013; Jentjens *et al.*, 2001; Rotman *et al.*, 2000; van Hall *et al.*, 2000; Wang *et al.*, 2015). Noticeably, five from the seven studies cited above examined whether the rate of muscle glycogen resynthesis by ingesting the proposed ‘optimal’ dose of carbohydrate (1.2 g·kg BM<sup>-1</sup>·h<sup>-1</sup>) can be exceeded with the addition of protein/amino acids, and none reported an accelerated rate of muscle glycogen resynthesis with protein co-ingestion (Beelen *et al.*, 2012; Detko *et al.*, 2013; Jentjens *et al.*, 2001; van Hall *et al.*, 2000; Wang *et al.*, 2015). An important distinction between the studies showing a glycogenic effect of protein co-ingestion and those with contradictory findings may be related to the precise amount of carbohydrate ingested during post-exercise recovery. When compiling the available data in humans (Figure 2.4), it becomes apparent that the ingestion of  $\approx 1.2$  g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup> is likely to maximise muscle glycogen resynthesis rate and further stimulating insulin with the addition of protein does not appear to influence glycogen storage (Beelen *et al.*, 2012; Detko *et al.*, 2013; Jentjens *et al.*, 2001; van Hall *et al.*, 2000; Wang *et al.*, 2015). In contrast, when  $\leq 0.8$  g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup> is ingested, the addition of protein with this relatively moderate amount of carbohydrate may enhance muscle glycogen resynthesis rates (Berardi *et al.*, 2006; Ivy *et al.*, 2002; van Loon *et al.*, 2000b; Williams *et al.*, 2003; Zawadzki *et al.*, 1992).



**Figure 2.4** Reported rates of muscle glycogen resynthesis across 17 different investigations that have measured muscle glycogen concentrations during short-term (2-6 h) recovery with varied rates of carbohydrate with or without protein in humans (Beelen *et al.*, 2012; Berardi *et al.*, 2006; Betts *et al.*, 2008; Carrithers *et al.*, 2000; Detko *et al.*, 2013; Ferguson-Stegall *et al.*, 2011b; Howarth *et al.*, 2009; Ivy *et al.*, 2002; Jentjens *et al.*, 2001; Lunn *et al.*, 2012; Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997; van Hall *et al.*, 2000; van Loon *et al.*, 2000b; Wang *et al.*, 2015; Williams *et al.*, 2003; Zawadzki *et al.*, 1992). The trend lines denote the suggested patterns of muscle glycogen resynthesis with each treatment (solid trend line represents carbohydrate ingestion while broken trend lines represent carbohydrate-protein ingestion).

It should be noted there are some inconsistent reports in the literature. For example, providing a carbohydrate dose of  $\leq 0.8$  g carbohydrate·kg BM<sup>-1</sup>·h<sup>-1</sup> with protein was shown to be ineffective in augmenting muscle glycogen relative to an energy-matched (Lunn *et al.*, 2012; Rotman *et al.*, 2000; Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997) or carbohydrate-matched supplement (Betts *et al.*, 2008). Although these discrepancies are not fully clear they may be related to differences in quantifying muscle glycogen, the provision of sub-optimal amounts of protein to stimulate insulin secretion or the specific type of exercise that was performed prior to recovery. Indeed, the study of Rotman *et al.* (2000) used <sup>13</sup>C-magnetic resonance spectroscopy to quantify glycogen, and while this method has been validated and shows a high correlation ( $r=0.95$ ;  $p<0.001$ ) with the needle biopsy technique (Taylor *et al.*, 1992), determining the rate of resynthesis is limited due to the absence of absolute glycogen concentrations. Additionally, the studies of Betts *et al.* (2008) and Lunn *et al.* (2012) employed an exercise protocol (i.e. running) that was not commonly used in the other cycling-based investigations. Indeed, it has been demonstrated that performing muscular contractions with an eccentric component such as running impairs both contraction (Kristiansen *et al.*, 1996) and insulin-induced glucose uptake by the muscle (Asp *et al.*, 1997). As such, eccentric exercise has been shown to impair muscle glycogen resynthesis (Costill *et al.*, 1990), which was corroborated by the study of Betts *et al.* (2008) as reflect by the relatively low rates of glycogen resynthesis in recovery ( $\approx 12$  mmol·kg dm<sup>-1</sup>·h<sup>-1</sup>). Of note, no study examined the role of an exhaustive prior running exercise bout on muscle glycogen resynthesis. This may be an important distinction, given that the magnitude of glycogen depletion influences the rate of muscle glycogen restoration during post-exercise recovery (Zachwieja *et al.*, 1991).

As mentioned in section 2.4.4.1, the addition of approximately 0.3-0.4 g·kg BM·h<sup>-1</sup> of protein may be required to achieve the synergistic effect of a carbohydrate-protein mixture on insulin secretion. In accordance, neither muscle glycogen storage nor insulin were significantly elevated when protein was included ( $\leq 0.2$  g·kg BM·h<sup>-1</sup>) in a carbohydrate-protein mixture relative to carbohydrate alone (Carrithers *et al.*, 2000; Lunn *et al.*, 2012; Roy and Tarnopolsky, 1998; Tarnopolsky *et al.*, 1997). These findings may help explain the lack of effect of protein co-ingestion when added to

relatively moderate amounts of carbohydrate. However, an accelerated rate of muscle glycogen resynthesis has been reported when modest amounts of protein ( $<0.2 \text{ g}\cdot\text{kg BM}\cdot\text{h}^{-1}$ ) were added to  $\approx 0.6\text{-}0.7 \text{ g carbohydrate}\cdot\text{kg BM}\cdot\text{h}^{-1}$  (Berardi *et al.*, 2006; Ivy *et al.*, 2002), despite the fact that no significant differences in insulin concentration were observed in the carbohydrate-protein treatment (Ivy *et al.*, 2002). This led authors of the latter study to speculate that alternative mechanisms may exist in relation to an accelerated muscle glycogen synthetic response. Unfortunately, the source of protein was not reported in that investigation, however, essential amino acids such as isoleucine and leucine may act in concert to facilitate glucose uptake and consequent incorporation into muscle glycogen independent of insulin (Bernard *et al.*, 2011; Doi *et al.*, 2005), presumably by an increased phosphorylation of AS160 signalling in the absence of insulin (Kleinert *et al.*, 2011).

The fate of glucose following carbohydrate-protein mixtures must be considered to assess the relevance of hyperinsulinaemia during post-exercise recovery on glucose disposal and subsequent incorporation as endogenous carbohydrate storage. In this regard, it is interesting to note that a significant inverse relationship ( $r = 0.99$ ;  $p < 0.001$ ) exists between the amount of protein intake and blood glucose concentration (Spiller *et al.*, 1987). In concurrence, the majority of investigations on post-exercise protein co-ingestion have reported lower blood glucose concentrations relative to a carbohydrate-only beverage (Carrithers *et al.*, 2000; Ferguson-Stegall *et al.*, 2011b; van Hall *et al.*, 2000; van Loon *et al.*, 2000b; Wang *et al.*, 2015; Zawadzki *et al.*, 1992). Nevertheless, whether this attenuated glycaemic response was due to a delayed glucose appearance into circulation or an increased glucose uptake by the muscle remains debatable. Although it is tempting to speculate that the lower glucose levels with protein co-ingestion are reflective of an increased glucose uptake secondary to insulin stimulation, there is some evidence to refute this contention. Thus, while the addition of protein to carbohydrate resulted in a  $\approx 100\text{-}190 \%$  higher insulin and  $\approx 35\text{-}42 \%$  lower glucose response, the rate of glucose disappearance using continuous glucose tracer infusions was identical between a carbohydrate control and the carbohydrate-protein mixtures (Kaastra *et al.*, 2006). The authors, however, noted a significant (12 %) reduced rate of glucose appearance, implying the lower glucose response with carbohydrate-protein mixtures may partly involve a delayed appearance

of glucose. Nevertheless, studies in rodents have demonstrated a hypoglycaemic effect of certain amino acids and consequent increased glucose uptake by the muscle (Bernard *et al.*, 2011; Doi *et al.*, 2005; Kleinert *et al.*, 2011). While the hypoglycaemic effect of a mixture of amino acids has been reported in humans, whether this is related to an increased glucose uptake remains unclear (Wang *et al.*, 2015). A number of possible mechanisms may therefore be attributed to the relatively delayed appearance of glucose in the latter study and while a slower gastric emptying and/or intestinal absorption may contribute to the delayed glucose appearance, it is unlikely to fully explain the lower glucose response following carbohydrate-protein ingestion (Kaastra *et al.*, 2006). An alternative explanation would be an insulin-induced suppression of hepatic glucose output, which is known to inhibit gluconeogenesis and glycogenolysis by  $\approx 55$  and 100 % when insulin is stimulated to approximately 450 pmol/L, respectively (Boden *et al.*, 2002). Indeed, insulin concentration in the study of Kaastra (2006) reached 480-700 pmol/L and would therefore be expected to exert an inhibitory effect on hepatic glucose output.

## **2.5 Restoration of exercise capacity following short-term recovery**

### **2.5.1 Effect of carbohydrate ingestion on time to exhaustion**

Given the intrinsic link between muscle glycogen depletion and endurance capacity, restoration of these endogenous carbohydrate stores is central to the recovery process (Ahlborg *et al.*, 1967; Betts *et al.*, 2007). While performance decrements and declined ability to maintain repeated intensified training may be the outcomes of insufficient glycogen repletion between exercise bouts during long term recovery (i.e.  $\geq 24$ -h) (Costill *et al.*, 1988; Nicholas *et al.*, 1997; Yeo *et al.*, 2008b) and that nutrition is inherently associated to this process, little is known regarding the optimal nutritional intervention that could translate into an enhancement in subsequent exercise capacity following short-term recovery. For example, it was shown that moderate to high-intensity ( $60 - 70 \% \dot{V}O_{2\max}$ ) endurance capacity can be improved when  $\approx 0.3 - 0.7 \text{ g.kg}^{-1}.\text{h}^{-1}$  of carbohydrate is ingested when compared to a placebo fluid (Bilzon *et al.*, 2000; Fallowfield *et al.*, 1995; Lee *et al.*, 2011). On the other hand, other studies found no effect of carbohydrate ingestion on subsequent cycling endurance capacity



(Casey *et al.*, 2000), Intermittent running endurance capacity (Taylor *et al.*, 2011) and cycling time trial performance (Ferguson-Stegall *et al.*, 2011b) when compared to a placebo beverage. Some of these paradoxical findings may be related to subtle differences in the adopted experimental protocols, such as measuring endurance capacity under warm environments (Bilzon *et al.*, 2000; Lee *et al.*, 2011) that could trigger a different mechanism to the onset of fatigue independent of substrate depletion (Nybo, 2010). Additionally, differences in feeding frequency may also contribute to the disparity between the studies through frequent (Betts *et al.*, 2007; Wong *et al.*, 2000) less frequent (Fallowfield and Williams, 1997; Fallowfield *et al.*, 1995; Ferguson-Stegall *et al.*, 2011b; Taylor *et al.*, 2011) or single bolus (Casey *et al.*, 2000) provisions of carbohydrate during the imposed recovery period. Nonetheless, there is evidence to suggest that frequency of carbohydrate intake during short-term recovery does not influence subsequent endurance capacity (Siu *et al.*, 2004). Indeed, the ambiguity of the efficacy of ingesting carbohydrate on subsequent endurance performance is present irrespective of the frequency of ingestion.

It can therefore be postulated that the ingestion of carbohydrate can enhance endurance capacity relative to a placebo. However, increasing the amount of carbohydrate by approximately threefold during limited recovery failed to yield further improvements in subsequent endurance capacity (Fallowfield and Williams, 1997; Wong and Williams, 2000), notwithstanding that a dose-dependent effect was reported in a later study (Betts *et al.*, 2007). Regardless of the fact that the exercise protocol was similar between those studies, the characteristics of participants were profoundly different. Specifically, lower blood lactate and higher  $\dot{V}O_{2\max}$  values were observed in the study of Betts and colleagues (2007) when compared to the other investigations (Fallowfield and Williams, 1997; Wong and Williams, 2000), indicative of a more aerobically trained sample in the former. Thus, training status may further explain the mixed results regarding endurance capacity following provisions of different amounts of carbohydrate, given that well-trained individuals who are familiarised with exercise capacity testing exhibit a more reliable reflection on performance measures (Hopkins *et al.*, 2001).

Another possible explanation for the discrepant findings between the study of Betts *et al.* (2007) and those that did not observe a dose-dependent effect on repeated exercise capacity (Fallowfield and Williams, 1997; Wong and Williams, 2000) may be related to the precise amount of carbohydrate that was ingested during recovery. It was shown that increasing carbohydrate during recovery from 0.15 to 0.53 g·kg BM<sup>-1</sup>·h<sup>-1</sup> did not elicit an improved on the capacity to run to exhaustion at 70 %  $\dot{V}O_{2\max}$  (Wong and Williams, 2000). These similar conditions were subsequently investigated by Tsintzas *et al.* (2003) to assess glycogen storage during recovery and its subsequent utilisation during a second bout. Although muscle glycogen resynthesis rates were  $\approx 250$  % greater when carbohydrate was ingested at a rate of 0.53 g·kg BM<sup>-1</sup>·h<sup>-1</sup>, glycogen utilisation during subsequent exercise was not different between treatments (Tsintzas *et al.*, 2003). These findings may suggest that glycogen content may not be the most important factor in restoring endurance capacity when the recovery period is limited. It should be recognised, however, that the second bout did not measure endurance capacity (i.e. the second run was a fixed duration of only 15 min), and that glycogen utilisation rates towards the end of an exhaustive bout may have differed between the trials. Furthermore, the amount provided in the latter study was much lower than the amount of carbohydrate suggested to maximise post-exercise glycogen resynthesis rates of  $\approx 1.2$  g·kg BM<sup>-1</sup>·h<sup>-1</sup> (Howarth *et al.*, 2009; van Loon *et al.*, 2000b). Thus, when ingesting carbohydrate at a rate that approaches the aforementioned recommended carbohydrate intakes to maximise muscle glycogen stores, an enhancement in endurance capacity was observed relative to modest lower amounts (0.8 *versus* 1.1 g·kg BM<sup>-1</sup>·h<sup>-1</sup>) of carbohydrate (Betts *et al.*, 2007). This may imply that increasing carbohydrate ingestion following a prior exercise bout is likely to increase muscle glycogen resynthesis during limited recovery, which in turn would result in an improvement in repeated exercise capacity. Nevertheless, no direct evidence of measuring muscle glycogen metabolism during a repeated exhaustive exercise bout makes it difficult to conclude whether maximising glycogen availability during limited recovery would influence the capacity for repeated exercise.

**Table 2.1** Effects of carbohydrate ingestion during short-term recovery on repeated exercise capacity.

Study	CHO intake (g·kg BM <sup>-1</sup> ·h <sup>-1</sup> )	Recovery time (h)	Timing of ingestion (min)	Mode of exercise	Repeated exercise protocol	Exercise capacity (min)
Fallowfield et al. (1995)	0, 0.5	4	IMPE, 120	Running	TTE @ 70% $\dot{V}O_{2\max}$	40, 62*
Fallowfield et al. (1997)	0.5, 1.5	4	IMPE, 120	Running	TTE @ 70% $\dot{V}O_{2\max}$	59, 58
Wong & Williams (2000)	0.15 <sup>†</sup> , 0.53	4	30, 60, 90, 120, 150	Running	TTE @ 70% $\dot{V}O_{2\max}$	65, 57
Wong et al. (2000)	0, 0.9	4	30, 60, 90, 120, 150, 180	Running	TTE @ 70% $\dot{V}O_{2\max}$	45, 69*
Bilzon et al. (2000)	0, 0.43	4	IMPE, 60, 120, 180	Running	TTE @ 60% $\dot{V}O_{2\max}$	45, 61*
Casey et al. (2000)	0, 0.25 G, 0.25 S	4	IMPE	Cycling	TTE @ 70% $\dot{V}O_{2\max}$	35, 40 G, 46 S
Betts et al. (2007)	0.8, 1.1	4	IMPE, 30, 60, 90, 120,150,180, 210	Running	TTE @ 70% $\dot{V}O_{2\max}$	84, 100*

G, glucose; S, sucrose; IMPE, immediately post-exercise; TTE, time to exhaustion; TT, time Trial; \*, significantly greater than other treatment(s) ( $p \leq 0.05$ ).

### 2.5.2 Effect of protein co-ingestion on time to exhaustion

The addition of protein to a carbohydrate supplement may accelerate the rate of muscle glycogen resynthesis (Berardi *et al.*, 2006; Ivy *et al.*, 2002; Zawadzki *et al.*, 1992). It would therefore be reasonable to suggest that protein-co-ingestion has the potential to improve subsequent endurance capacity, given the relationship between pre-exercise muscle content glycogen and exercise time to exhaustion (Bergstrom *et al.*, 1967). In this regard, the restoration of muscle glycogen during limited recovery is considered a possible mechanism for the ergogenic effect of carbohydrate-protein ingestion on repeated exercise and thus glycogen restoration will only be discussed in relevance to subsequent endurance capacity in this section. Moreover, the interaction of ingested amino acids with the liver may also be relevant for short-term recovery, as liver glycogen resynthesis appears to be an important factor affecting subsequent exercise. Some support of this notion can be obtained when considering the correlation between the recovery of exercise capacity and the restoration of bodily endogenous carbohydrate stores (muscle and liver glycogen;  $r = 0.55$ ;  $p < 0.05$ ) relative to restoration of hepatic glycogen ( $r = 0.53$ ;  $p < 0.05$ ) stores alone (Casey *et al.*, 2000). However, paucity of information exists in relation to the effects of protein co-ingestion on repeated exercise capacity.

In fact, very few studies directly measured the rate of glycogen resynthesis during the recovery phase and subsequent endurance capacity (Lunn *et al.*, 2012; Williams *et al.*, 2003) or performance (Ferguson-Stegall *et al.*, 2011b) with protein co-ingestion. Notwithstanding that the study of Williams and colleagues (2003) employed independent sub-groups to separately examine the role of carbohydrate-protein on glycogen resynthesis and subsequent endurance capacity, the authors showed a 55 % improvement in cycling time to exhaustion at 85 %  $\dot{V}O_{2\max}$  when protein was added to a carbohydrate supplement. Nonetheless, the experimental design of that study failed to demonstrate whether the improvements were attributed to the protein fraction *per se* or the 167 % increase in caloric intake; an important factor in determining the rate of muscle glycogen resynthesis during post-exercise recovery (Roy and Tarnopolsky, 1998). The provision of these two supplements at a similar rate of ingestion were investigated to determine the restoration of exercise capacity and

reported that cycling capacity may actually be impaired with the inclusion of protein (Karp *et al.*, 2006), albeit a milk-based carbohydrate-protein mixture did not show these negative effects (Thomas *et al.*, 2009).

Regardless of these limitations, the findings of Williams *et al.* (2003) provide intriguing evidence that repeated exercise capacity may be enhanced with the presence of protein or with increasing energy intake in a dose-dependent manner. A more recent investigation accounted for the caloric equivalency when comparing a carbohydrate-protein as opposed to an isocaloric carbohydrate beverage on recovery rates and repeated exercise capacity (Lunn *et al.*, 2012). Although no differences were noted in muscle glycogen resynthesis during 3 h of recovery, subsequent endurance capacity was significantly improved with the ingestion of the carbohydrate-protein mixture (Lunn *et al.*, 2012). It is noteworthy that the beneficial outcomes for protein intake in this study cannot be solely attributed to the protein fraction, as the study used chocolate milk that includes other nutrients that may affect glycogen storage and/or subsequent performance such as caffeine (Pedersen *et al.*, 2008). Furthermore, the study utilised a capacity test that induced fatigue within ~3 min that may suggest that other factors other than glycogen-dependent mechanisms were responsible for the termination of exercise (Betts *et al.*, 2005).

Another study of relevance when examining repeated exercise following limited recovery is the study by Ferguson-Stegall *et al.* (2011b). In concurrence with the many of the studies in the literature, when supplements were matched for energy content and provided in optimal amounts (i.e.  $\geq 1 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ), protein did not appear to augment glycogen resynthesis beyond ingesting carbohydrate (Ferguson-Stegall *et al.*, 2011b). Of note, the aforementioned study did not report absolute glycogen concentrations during recovery and hence limits the interpretation of this data. Notwithstanding this evidence, repeated cycling performance was shown to improve beyond that of an isocaloric carbohydrate following the ingestion of a milk-based carbohydrate-protein mixture (Ferguson-Stegall *et al.*, 2011b) lending support to the notion that improvements in subsequent exercise may be unrelated to muscle glycogen resynthesis during short-term recovery. With these factors in mind, the

addition of protein to a carbohydrate supplement may exert an ergogenic effect on repeated exercise capacity/performance irrespective of the underlying physiological mechanism of this effect.

Further studies investigated the efficacy of protein feeding during the limited recovery period on subsequent endurance capacity (Betts *et al.*, 2007; Betts *et al.*, 2005; Millard-Stafford *et al.*, 2005) and performance (Berardi *et al.*, 2008; Berardi *et al.*, 2006; Morifuji *et al.*, 2012; Richardson *et al.*, 2012) independent of the assessment of glycogen concentrations following an initial exercise bout. The findings of these investigations are inconsistent with some showing ergogenic effects of acute carbohydrate-protein feeding of both the capacity to sustain endurance exercise (Betts *et al.*, 2007) and performance (Berardi *et al.*, 2008), while others did not reach similar conclusions (Berardi *et al.*, 2006; Betts *et al.*, 2005; Millard-Stafford *et al.*, 2005; Morifuji *et al.*, 2012; Richardson *et al.*, 2012). Similar to the nutritional considerations regarding muscle glycogen resynthesis, the precise amount of ingested carbohydrate and whether the supplements were matched for energy content may provide a possible explanation for these discrepant findings. Of important relevance is the study by Betts *et al.* (2007), which demonstrated that the addition of protein ( $0.3 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) to a carbohydrate supplement  $0.8 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) restored the capacity for repeated exercise more completely than when a carbohydrate-matched supplement was ingested. However, recovery of exercise capacity was restored to a similar magnitude in the carbohydrate-protein mixture when compared with an isocaloric carbohydrate supplement (Betts *et al.*, 2007).

These findings clearly demonstrate that the addition of protein can enhance repeated exercise capacity when increasing the caloric content of a carbohydrate supplement, and that carbohydrate intake should be  $\geq 1.1 \text{ g carbohydrate} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  to allow greater restoration of exercise capacity. Interestingly, these identical nutritional provisions were reported in a subsequent study by the same authors and reported no acceleration of muscle glycogen resynthesis between a carbohydrate-protein mixture and a control solution of matched carbohydrate content (Betts *et al.*, 2008). This provides further indication that enhancement in repeated exercise can occur with

carbohydrate protein ingestion independent of muscle glycogen resynthesis. Rather, a consistent finding was related to an increased rate of whole-body carbohydrate oxidation with carbohydrate-protein ingestion (Betts *et al.*, 2007; Betts *et al.*, 2008). Coupled with the fact that glycogen degradation was similar between a carbohydrate-matched control beverage and carbohydrate-protein mixture (Betts *et al.*, 2008), it is reasonable to suggest that an improved maintenance of euglycaemia and/or increased extra-muscular carbohydrate oxidation may explain, at least in part, the ergogenic effect of protein co-ingestion during recovery.

Considering other potential mechanisms for the ergogenic effect for the addition of protein warrants discussion, particularly given the reported dissociation between the restoration of muscle glycogen and repeated exercise capacity/performance in some (Ferguson-Stegall *et al.*, 2011b; Lunn *et al.*, 2012) but not all (Williams *et al.*, 2003) studies. It was previously proposed that the addition of protein may provide precursors for *de novo* synthesis of tricarboxylic acid cycle intermediates and thus may enable anaplerotic replenishment of tricarboxylic acid cycle flux in the skeletal muscle (Wagenmakers, 1998). While a decline in tricarboxylic acid cycle intermediate pool was shown during prolonged exercise, aerobic provision was not compromised as evidenced by stable limb oxygen uptake during exercise and no change in muscle phosphocreatine concentration, which is a sensitive indicator of mitochondrial respiration (Gibala *et al.*, 2002). It was therefore concluded from the latter study that changes in muscle tricarboxylic acid cycle intermediates are not causally related to the capacity for aerobic energy provision during prolonged exercise. Another proposed mechanism for the ergogenic effect of protein co-ingestion may be related to the role of amino acids in brain function and postponement of central fatigue (Meeusen, 2014). Although there is some evidence to suggest that the ingestion of protein or amino acids reduces perceptions of fatigue during exercise (Alghannam, 2011; Blomstrand *et al.*, 1997), it remains debatable whether the inclusion of protein with carbohydrate can improve exercise performance through attenuated sensation of fatigue (Madsen *et al.*, 1996; van Hall *et al.*, 1995). Interestingly, a recent study in rodents reported that the co-ingestion of protein with carbohydrate attenuates skeletal muscle glycogen depletion during exercise (Morifuji *et al.*, 2011). The latter study demonstrated that pre-exercise ingestion of glucose

plus whey protein hydrolysate caused an attenuation in muscle glycogen depletion during a subsequent exercise, which was concomitant with an activation of key enzymes that regulate glucose uptake and glycogen synthesis (Akt, PKC and glycogen synthase) during exercise relative to water ingestion (Morifuji *et al.*, 2011). Thus, the possibility of protein to attenuate glycogen degradation or increase the net balance of glycogen metabolism (an increase in the ratio of glycogen synthesis and degradation) may be a candidate for the ergogenic effects of protein co-ingestion. Unfortunately, however, no study examined the effects of carbohydrate-protein mixture on glycogen depletion during a repeated exhaustive exercise bout in humans.



**Table 2.2** Effects of carbohydrate-protein ingestion during short-term recovery on repeated exercise capacity.

Study	CHO intake (g·kg BM <sup>-1</sup> ·h <sup>-1</sup> )	Pro intake (g·kg BM <sup>-1</sup> ·h <sup>-1</sup> )	Recovery time (h)	Timing of ingestion (min)	Mode of exercise	Repeated exercise protocol	Exercise capacity (min)
Williams et al. (2003)	0.15, 0.4	0, 0.1	4	IMPE, 120	Cycling	TTE @ 85% $\dot{V}O_{2\max}$	20, 31 <sup>*</sup>
Millard-Stafford et al. (2005)	0.6, 1.0, 0.8	0, 0, 0.2	2	IMPE, 60	Running	TTE @ 90% $\dot{V}O_{2\max}$	6, 6, 5
Betts et al. (2005)	0.8, 0.8, 1.2, 1.2	0, 0.1, 0, 0.2	4	IMPE, 30, 60, 90, 120, 150, 180, 210	Running	TTE @ 85% $\dot{V}O_{2\max}$	18, 20, 15, 18
Karp et al. (2006)	0.2, 0.5, 0.5 <sup>†</sup>	0, 0.13, 0.13 <sup>†</sup>	4	IMPE, 120	Cycling	TTE @ 70% $\dot{V}O_{2\max}$	41 <sup>*</sup> , 29, 40 <sup>†*</sup>
Betts et al. (2007)	0.8, 1.1, 0.8	0, 0, 0.3	4	IMPE, 30, 60, 90, 120, 150, 180, 210	Running	TTE @ 70% $\dot{V}O_{2\max}$	84, 91 <sup>*</sup> , 100 <sup>*</sup>
Thomas et al. (2009)	0.2, 0.5, 0.4 <sup>†</sup>	0, 0.13, 0.10 <sup>†</sup>	4	IMPE, 120	Cycling	TTE @ 70% $\dot{V}O_{2\max}$	23, 21, 32 <sup>†*</sup>
Lunn et al. (2012)	0.25, 0.20 <sup>†</sup>	0, 0.05 <sup>†</sup>	3	IMPE	Running	TTE @ incline achieved at $\dot{V}O_{2\text{ peak}}$	3, 4 <sup>†*</sup>
Richardson et al. (2012)	1.5, 1.2	0, 0.3	3	IMPE, 30, 60, 90, 120	Cycling	TTE @ 75% $\dot{V}O_{2\max}$	25, 24

IMPE, immediately post-exercise; R, running; C, cycling; TTE, time to exhaustion; TT, time Trial; †, provided in the form of chocolate milk; \*, significantly greater than other treatment(s) ( $p \leq 0.05$ ).

## 2.6 Endurance training adaptations

A central aim for participants involved in endurance-type events is to improve their capacity to sustain a desired speed or power output. Training to enhance endurance performance includes a number of physiological and metabolic adaptations that improve the resistance of working muscles to fatigue secondary to the continuous overload of the training stimulus. When this training stimulus is conducted over extended periods of time, chronic adaptations occur. It has been suggested that adaptation to exercise is a consequence of the amalgamation of acute exercise bouts (Coffey *et al.*, 2011; Egan and Zierath, 2013; Hawley *et al.*, 2011). In line with this reasoning, the acute signalling response following each distinct exercise bout can be considered as the initial step leading to gradual changes in protein content and enzyme activity that modulate chronic adaptations (Egan and Zierath, 2013; Hawley *et al.*, 2011).

The extent to which substrate availability influences the acute response to an exercise bout has been an expanding research area in recent years. Indeed, nutrient availability was shown to influence substrate selection/fuel utilisation during exercise, amplify the transcriptional activity of several metabolic genes and enhance the phosphorylation of key signalling proteins (Coffey *et al.*, 2011; Hansen *et al.*, 2005; Hulston *et al.*, 2010; Pilegaard *et al.*, 2002). Therefore, nutrient-exercise interactions may have an important role in enhancing the magnitude of training adaptations. This section will place an emphasis on the effects of post-exercise protein ingestion upon modifying the training stimulus in relation to endurance exercise.

### 2.6.1 Cardiovascular training adaptations

A fundamental adaptation to prolonged endurance exercise such as running and cycling is an improved oxygen transport capacity of the cardiovascular system and by virtue of these changes the maximal capacity for oxygen consumption is enhanced (Clausen, 1977). Along with systemic changes, for instance left ventricular dilatation and a reduction in both resting and maximal heart rate, cardiovascular adaptations involve an increase in plasma volume and thus lower blood viscosity (Goodman *et al.*, 2005; Rivera-Brown and Frontera, 2012). These responses enhance stroke volume and cardiac output, which consequently improve oxygen

carrying capacity by the circulatory system (Clausen, 1977; Ekblom *et al.*, 1968; Fellmann, 1992). Coupled together, these systemic and peripheral physiological adaptations can induce improvements in maximal oxygen uptake, a well-established indicator of cardiorespiratory fitness and health (Åstrand *et al.*, 2003).

Endurance exercise training is known to increase blood volume, and this term comprises the sum of plasma and erythrocyte volumes (Sawka *et al.*, 2000). Of these two constituents, however, it appears that plasma volume expansion is the principal contributor to the increase in blood volume (Sawka and Coyle, 1999). Numerous investigations reported an increase in plasma volume following endurance training (Convertino *et al.*, 1980; Goodman *et al.*, 2005; Green *et al.*, 1991; Mier *et al.*, 1996), but this is not without exception (Ray *et al.*, 1990). The latter inconsistency, however, can be ascribed to differences in posture (i.e. supine *versus* standing endurance training), which along with other factors (for example exercise intensity/duration, training status and environmental stress) has been implicated to influence plasma volume expansion (Fellmann, 1992; Greenleaf *et al.*, 1979). Notwithstanding this, the overwhelming evidence supports the notion that blood volume increases following aerobic training and that plasma volume expansion is the likely mechanism for this phenomenon (Sawka *et al.*, 2000; Sawka and Coyle, 1999).

The expansion of plasma volume is predominantly dependent upon the increase in total plasma protein content (Gillen *et al.*, 1991; Haskell *et al.*, 1997). Namely, the content of plasma albumin was shown to account for 86 % of the increase in total plasma protein content in young healthy individuals (Convertino *et al.*, 1980). Albumin is synthesised in the liver and is responsible for  $\approx 75$  % of the colloid oncotic pressure of plasma causing fluid retention in the vasculature and consequently can induce hypervolaemia (Convertino *et al.*, 1980). Interestingly, a significant correlation between the change in training-induced plasma volume and albumin content has been reported ( $r^2 = 0.69$ ;  $p = 0.05$ ) indicating that chronic hypervolemia is oncotically mediated (Okazaki *et al.*, 2009b). Given that nutritional intake, specifically protein, may increase plasma albumin concentration and hence affect the post-exercise hypervolaemic response (Moore *et al.*, 2009; Thalacker-Mercer *et al.*, 2007), an intriguing extension to this knowledge is the potential for the addition of protein following

exercise may enhance the magnitude of training response through albumin-mediated mechanisms. This possibility is further elaborated in section 2.6.3.

The ingestion of protein following exercise presents a dose-response relationship with plasma albumin fractional synthetic rate, such that the latter increases twofold with the ingestion of 20 g of protein relative to when no protein was ingested ( $0.4 \% \cdot h^{-1}$  versus  $0.2 \% \cdot h^{-1}$ ) (Moore *et al.*, 2009). Following an acute prolonged exercise bout at 70-80 %  $\dot{V}O_{2max}$ , the concentration of plasma albumin declines by  $\approx 0.08 \text{ g} \cdot \text{kg}^{-1}$  and in contrast to a placebo, the ingestion of post-exercise carbohydrate-protein ingestion restores plasma albumin content to baseline levels during short-term (5 h) recovery. Concomitantly, the ingestion of carbohydrate-protein was shown to further increase plasma albumin content, albeit to a smaller magnitude, by  $0.02 \text{ g} \cdot \text{kg}^{-1}$ , while no changes from baseline were shown in the placebo trial (Okazaki *et al.*, 2009a). Moreover, the acute responses with post-exercise protein ingestion were shown to mediate chronic changes in plasma albumin content (by  $\approx 0.6 \text{ g} \cdot \text{kg}^{-1}$ ) following eight weeks of endurance exercise training (Okazaki *et al.*, 2009b).

### 2.6.2 Intramuscular training adaptations

The ability to perform prolonged submaximal exercise not only relies on the ability of the cardiovascular system to transport oxygen to the working muscle. Of equal importance is the capacity to extract and utilise the oxygen from the arterial blood by the contracting muscle (Rivera-Brown and Frontera, 2012). In this regard, skeletal muscle is a highly malleable tissue that is capable to undergo major adaptations to exercise training (Holloszy, 2008). Prolonged endurance training elicits a number of morphological and metabolic adaptations that include fibre-specific (from a predominantly glycolytic fast to a predominantly oxidative slow-twitch) transformation (Zierath and Hawley, 2004), changes in substrate utilisation favouring glycogen sparing (Hulston *et al.*, 2010; Leblanc *et al.*, 2004; Talanian *et al.*, 2010) and increased mitochondrial density (Hood, 2001).

Endurance exercise induces a number of contraction-induced stressors (e.g. increased AMP:ATP ratio,  $\text{Ca}^{2+}$  flux and decreased energy availability) that activate primary cell signalling kinases, such as CaMKII, p38MAPK and AMPK and subsequently activate

downstream transcription factors and co-activators that regulate mitochondrial protein content (Bartlett *et al.*, 2015). The increase in exercise-induced mitochondrial content and density is generally referred to as “mitochondrial biogenesis” and is the dominant adaptation to increase the maximal capacity of the muscle to generate ATP through oxidative phosphorylation (Hood *et al.*, 2006). Within six weeks of endurance training, mitochondrial density was shown to increase by 50 %-100 % (Hood, 2001). However, with a protein half-life of approximately 1 week for mitochondrial proteins (Henriksson and Reitman, 1977), a continuous training stimulus is needed to maintain the exercise-induced elevation in mitochondrial content. While the process of mitochondrial biogenesis is complex and highly regulated (Holloszy, 2008), peroxisome proliferator-activated receptor gamma co-activator-1 $\alpha$  (PGC-1 $\alpha$ ) is proposed to be a master regulator of mitochondrial biogenesis (Hood *et al.*, 2006). PGC-1 $\alpha$  mediates its effect by attaching and activating transcription factors that induce the expression of mitochondrial transcription factor A, and thus activating the expression of mitochondrial proteins (Lin *et al.*, 2005). Indeed, transient increases in PGC-1 $\alpha$  transcriptional activity can be observed after a single bout of exercise, and four weeks of endurance training were shown to further amplify this response (Pilegaard *et al.*, 2003).

With regards to nutrient availability, there is some evidence to support the notion that reduced carbohydrate availability (commencing some training sessions with reduced glycogen stores) in conjunction with endurance training may enhance endurance markers of mitochondrial activity such as citrate synthase activity (Hansen *et al.*, 2005; Yeo *et al.*, 2008b). However, it appears that post-exercise carbohydrate restriction does not influence the expression of PGC-1 $\alpha$  (Cochran *et al.*, 2010; Jensen *et al.*, 2015; Mathai *et al.*, 2008), suggesting that the mechanism by which skeletal muscle oxidative capacity is upregulated by aerobic exercise when combined with reduced carbohydrate availability may reside upstream of PGC-1 $\alpha$  and is dependent on AMPK and p38MAPK activation (Margolis and Pasiakos, 2013). When considered collectively, it appears that while PGC-1 $\alpha$  is a central regulator of mitochondrial biogenesis in response to endurance exercise, the precise mechanism by which reduced carbohydrate availability may mediate mitochondrial adaptation to endurance exercise remain unclear (Bartlett *et al.*, 2015).

To summarise, mitochondrial biogenesis is a central adaptation to endurance training, and PGC-1 $\alpha$  is well documented as master regulator for this process. While it is clear that endurance exercise mediates transient increases in PGC-1 $\alpha$  that over time increase mitochondrial protein content and thus training adaptations (Egan and Zierath, 2013), the role of carbohydrate availability in mediating mitochondrial biogenesis is relatively unknown although this may be related to AMPK, p38MAPK and/or p53 phosphorylation (Bartlett *et al.*, 2015). Another potential nutrient that may interact with the intramuscular adaptive response to endurance exercise is protein, which will be discussed in the next section.

### **2.6.3 Protein ingestion and endurance training adaptations**

As stated in section 2.6, adaptation to exercise is thought to occur as a consequence of the accumulated response of acute exercise bouts, while nutrient availability can influence the acute response to exercise and may modulate the chronic adaptive response to exercise training (Hawley *et al.*, 2011). Therefore, nutritional strategies provided in close temporal proximity to exercise have the potential to improve training efficiency by enhancing the magnitude of adaptations to the same training stimulus (van Loon, 2014). Emerging acute mechanistic evidence supports the potential benefit of post-exercise protein feeding in increasing muscle protein synthesis and mitigating proteolysis associated with an endurance exercise bout (Breen *et al.*, 2011; Howarth *et al.*, 2009; Lunn *et al.*, 2012). Cycling-based endurance training studies have also proposed the role of protein ingestion in supporting tolerance to intensified training in concurrence with an increase in magnitude of training adaptations when repeated bouts of exercise are performed (Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b; Witard *et al.*, 2011). Taken together, post-endurance exercise protein intake may provide means to facilitate aerobic training adaptations. Nonetheless, the time course effects of prolonged post-exercise protein co-ingestion in enhancing the adaptive response to running-based endurance exercise training remains to be established.

It is becoming clear that both the exercise stimulus and protein feeding act independently and synergistically in modulating muscle protein synthesis and consequently these subtle changes in muscle quantity or quality can mediate worthwhile training adaptations if sustained for weeks or months (Betts and Williams, 2010; Hawley *et al.*, 2011). Furthermore, protein ingestion during and following an acute endurance exercise bout has been shown to increase

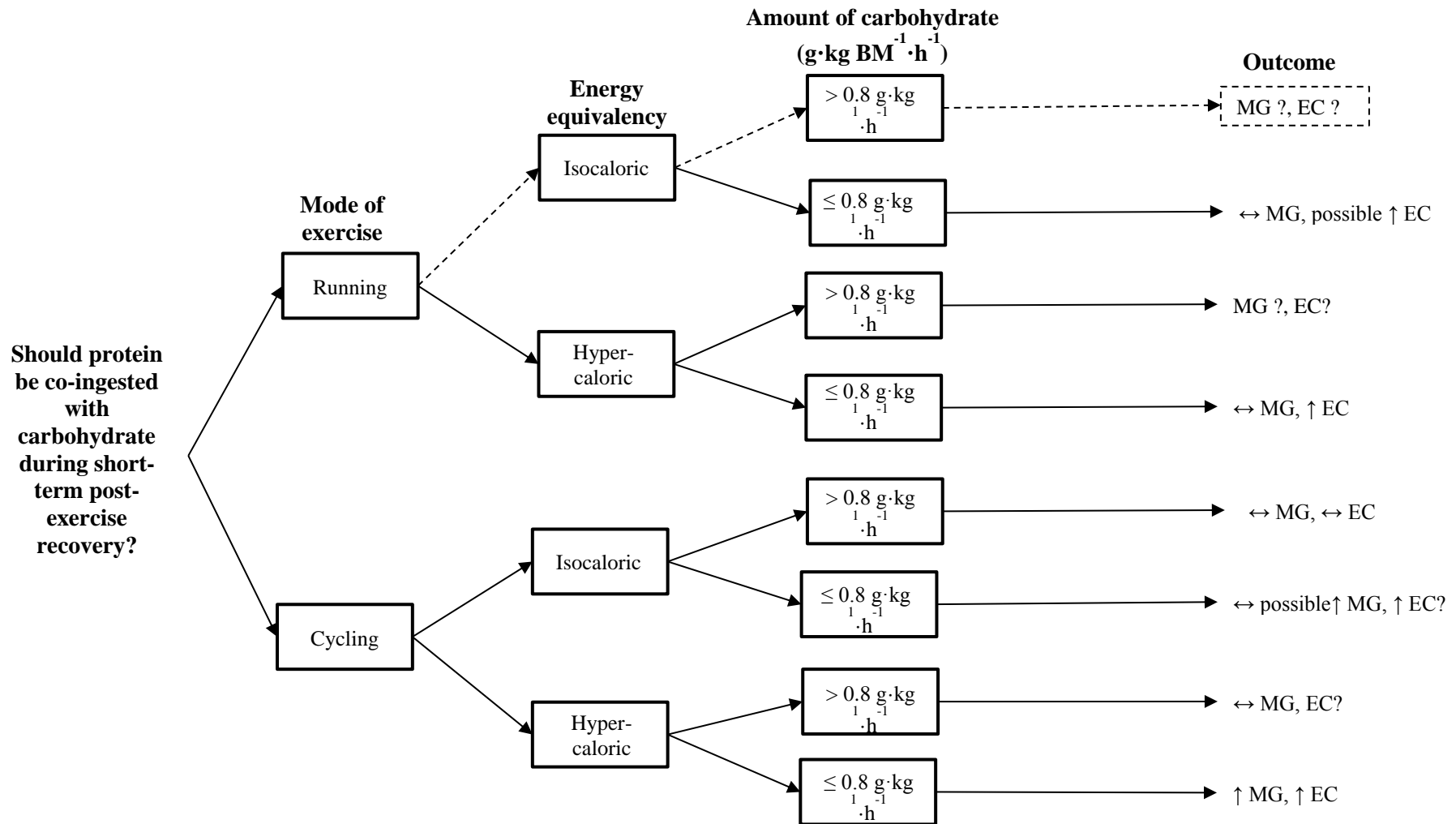
muscle protein synthesis, reduce muscle protein breakdown and thus results in an increased whole body net protein balance (Howarth *et al.*, 2009; Pasiakos *et al.*, 2011). Despite that endurance exercise does not typically result in muscle mass accrual, the changes in muscle protein synthesis following endurance exercise are relevant to drive tissue repair and remodelling in concurrence with the synthesis of non-contractile proteins such as the mitochondria (Burd *et al.*, 2009). Indeed, acute and chronic endurance exercise has been demonstrated to stimulate the mitochondrial protein synthetic response (Di Donato *et al.*, 2014; Wilkinson *et al.*, 2008) and this was shown to be stimulated by the availability of extracellular amino acids in a dose-response manner, at least in rest (Bohe *et al.*, 2003). Nonetheless, post-exercise protein ingestion appears to favour myofibrillar and not mitochondrial protein synthesis (Breen *et al.*, 2011). This may infer that exogenous protein availability can enhance the capacity for contractile muscular adaptations, although the role of post-exercise protein ingestion in enhancing the effects of running-based endurance exercise training remains to be established (Ferguson-Stegall *et al.*, 2011a; Moore and Stellingwerff, 2012).

An enhancement in oxidative capacity and maximal oxygen uptake in response to aerobic training with post-exercise protein ingestion has been observed by some (Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b) but not all (Cramer *et al.*, 2012) studies. In concordance, the role of protein feeding in enhancing the functionally adaptive response to endurance training may not reside in the intramuscular milieu. Rather, noticeable cardiovascular improvements in maximal oxygen uptake via an increase in plasma volume and plasma albumin content have been shown with protein ingestion (Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b). However, the addition of other nutrients such as caffeine, flavonoids, multivitamins and ribose (Cramer *et al.*, 2012; Ferguson-Stegall *et al.*, 2011a) alongside the absence of macronutrient-specific comparisons by not including a carbohydrate-only supplement (Okazaki *et al.*, 2009b) makes it difficult to conclude whether these improvements in training adaptation are a result of protein intake *per se*. Interestingly, the magnitude of improvement in maximal oxygen uptake during endurance training was greater when isolated whey protein was ingested relative to a placebo (Okazaki *et al.*, 2009b) or when milk-based protein available in the form of chocolate milk was co-ingested when compared to an energy matched carbohydrate supplement and a placebo (Ferguson-Stegall *et*

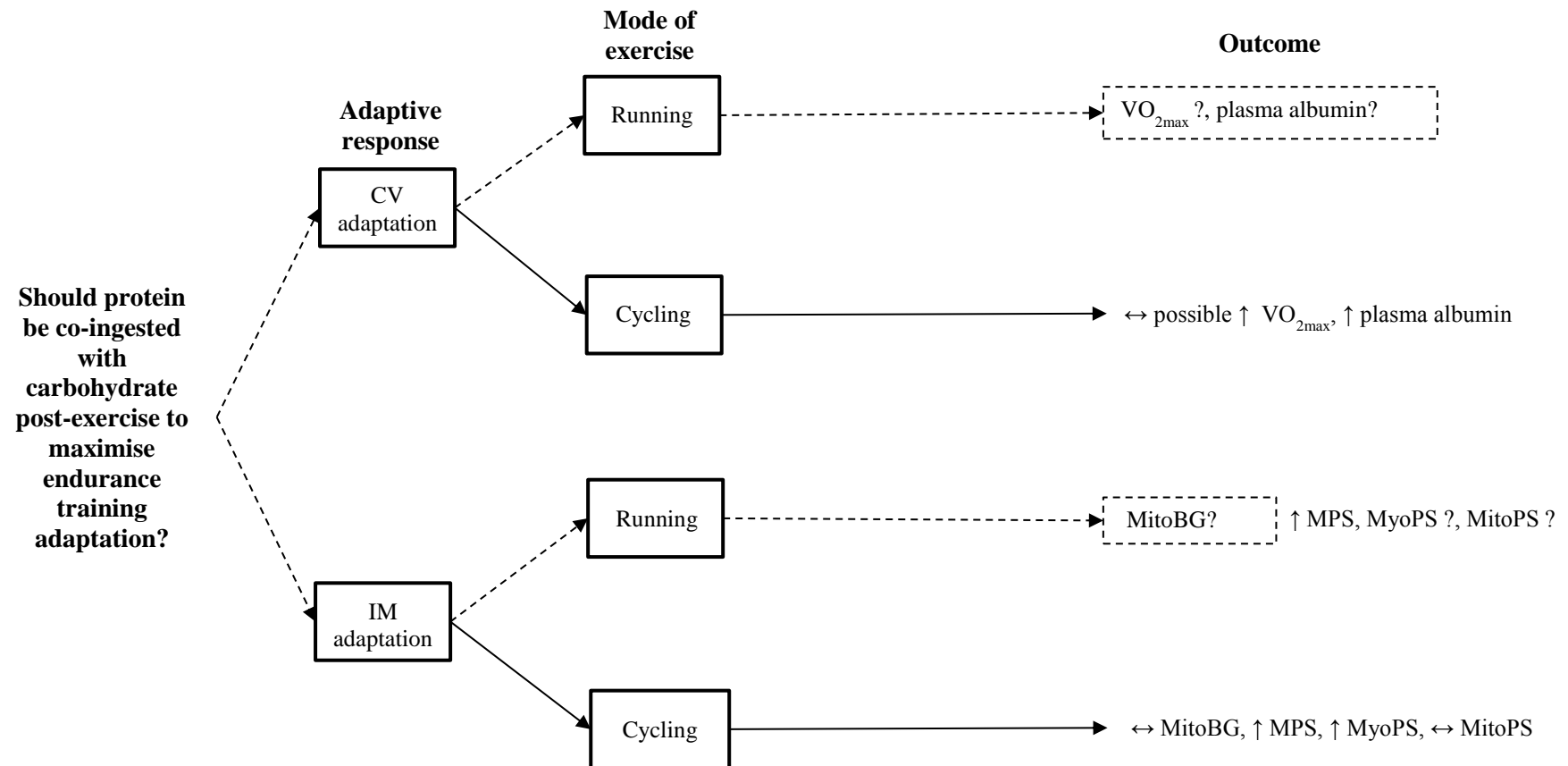
*al.*, 2011a). Collectively, these findings may infer nutrient-specific effects of post-exercise supplementation on endurance training adaptations.

To summarise, recent scientific evidence from acute laboratory investigations and relatively extended cycling-based training studies supports the notion that nutrient intake can increase protein accretion and ultimately this may influence the magnitude of the training effect. This nutrient-exercise interaction may modulate the adaptive response to training and protein feeding appears to be an important factor in mediating this process. However, examining the long-term outcomes of these acute mechanistic models, particularly during free living endurance-type exercise, require further investigation.





**Figure 2.5** Flow diagram illustrating the influence of post-exercise protein ingestion during short-term recovery on muscle glycogen resynthesis (Betts *et al.* 2008, Lunn *et al.* 2012) and restoration of endurance capacity (Betts *et al.* 2005, Millard-Stafford *et al.* 2005, Betts *et al.* 2007) from running and on muscle glycogen resynthesis (Zawadzki *et al.* 1992, Tarnopolsky *et al.* 1997, van Loon *et al.* 2000b, Carrithers *et al.* 2000, van Hall *et al.* 2000; Jentjens *et al.* 2001, Ivy *et al.* 2002, Williams *et al.* 2003, Berardi *et al.* 2006, Howarth *et al.* 2009, Ferguson-Stegall *et al.* 2011b, Beelen *et al.* 2012, Detko *et al.* 2013) and restoration of endurance capacity (Williams *et al.* 2003, Karp *et al.* 2006, Thomas *et al.* 2009, Richardson *et al.* 2012) from cycling. Dashed lines represent what remains to be established and aimed to be explored in this thesis. MG; muscle glycogen resynthesis, EC; endurance capacity.



**Figure 2.6** Flow diagram illustrating the influence of post-exercise protein ingestion on intramuscular (Howarth *et al.* 2009, Breen *et al.* 2011, Ferguson-Stegall *et al.* 2011a) and cardiovascular (Okazaki *et al.* 2009b, Ferguson-Stegall *et al.* 2011a, Robinson *et al.* 2011, Cramer *et al.* 2012) cycling endurance training adaptation, in addition to intramuscular (Lunn *et al.* 2012) running endurance training adaptation. Dashed lines represent what remains to be established and aimed to be explored in this thesis. CV; cardiovascular, IM; intramuscular, MPS; mixed muscle protein synthesis, MyoPS; myofibrillar protein synthesis, MitoPS; mitochondrial protein synthesis, MitoBG; mitochondrial biogenesis.

## 2.7 Summary

The notion that muscle glycogen is central to recovery is based on the plethora of evidence demonstrating a causal relationship between muscle glycogen content during an initial prolonged moderate to high intensity exercise and the onset of fatigue. Thus, previous research focused on the effects on different nutritional interventions to maximise the availability of this substrate, albeit the precise nutrient amount/composition remains debatable. Moreover, the current nutritional guidelines to acutely increase muscle glycogen content during short-term recovery are thought to improve repeated exercise capacity under the assumption that similar fatigue mechanisms operate during a subsequent exercise bout. However, no study has directly examined muscle glycogen metabolism during a repeated exhaustive exercise bout.

The co-ingestion of protein with carbohydrate during post-exercise recovery has received much attention in the last two decades. This can be ascribed to claims that addition of protein augments muscle glycogen resynthesis during short-term recovery and improves subsequent endurance capacity. Nevertheless, a number of confounding variables such as different caloric contents of the test beverages and the inclusion of other nutrients makes it difficult to establish the precise role of protein on muscle glycogen restoration. Furthermore, the suggestion that the addition of protein to a carbohydrate supplement improves subsequent exercise capacity is argued, albeit a physiological mechanism to this effect remains to be established. Although a reduction in muscle glycogen use was proposed as a candidate for this ergogenic effect, no study explored glycogen metabolism following protein co-ingestion with carbohydrate during a repeated exercise bout in humans.

In addition to the restoration of endogenous carbohydrate stores, post-exercise recovery includes a number of other important aspects such as rehydration and muscle tissue reconditioning. The latter is mainly dependent on the availability of sufficient protein to stimulate muscle protein synthesis and initiate tissue repair and remodelling and ultimately modulate the adaptive response to training. While post-exercise protein

ingestion has been shown to increase mixed muscle protein synthesis following a single exercise bout, very few investigations have explored the effects of long-term (i.e. weeks of training rather than a single bout) post-exercise protein supplementation on the adaptive response to endurance training, and none examined this using treadmill based exercise in young individuals.

The work in this thesis therefore aims to add to existing knowledge by, i) exploring the influence of muscle glycogen availability on a repeated exercise bout following short-term recovery, ii) the effect of carbohydrate-protein ingestion on muscle glycogen resynthesis following exhaustive running and its influence on muscle glycogen degradation during a repeated exercise bout, iii) the reliability of prolonged treadmill running as a measure of human endurance capacity in endurance-trained individuals and, iv) the effect of post-exercise protein co-ingestion on the adaptive response to six weeks of treadmill-based endurance training.

## CHAPTER 3

### GENERAL METHODS

#### 3.1 Introduction

This section provides generic information regarding the methodology adopted in **Chapters 4-6**. The umbrella project for these chapters was allocated the title ‘Macronutrient ingestion, Muscle glycogen and Post-exercise recovery’. The project was approved by the National Health Service (NHS) South West 3 Research Ethics Committee (REC) and was subsequently registered as a clinical controlled trial (ISRCTN 87937960).

All measurements were conducted at the Physiology Research Laboratory in the University of Bath. Recruitment of participants was achieved by means of advertisement and personal communication within the University campus and surrounding sporting clubs. The chosen target population were healthy non-smoking recreationally active males and females who include endurance training in the form of running as a central component ( $\geq 2$  h per week) of their training regime. The chosen age range for participation in the studies was 18-48 years old. Upon volunteering, the participants were initially briefed in writing followed by a verbal explanation of the protocol and the pre-requisites set for satisfactory inclusion on their first visit to the laboratories to ensure their full understanding before taking part in the investigation.

#### 3.2 Trial objectives

This research trial was aimed to investigate the relationships between nutrient intake, muscle glycogen metabolism and exercise capacity during a repeated bout of physical exercise. This involved an exploration of the effect of carbohydrate ingestion during short-term recovery upon muscle glycogen availability and/or subsequent endurance capacity, and whether this is linked to the carbohydrate fraction *per se* or simply to an energy intake surplus. In accordance, a replacement of a fraction of the carbohydrate

with a different macronutrient (whey protein hydrolysate) matched in energy content was examined to assess if whey protein hydrolysate co-ingestion provides a distinct benefit over carbohydrate alone in relation to muscle glycogen storage and/or the capacity for subsequent exercise. The trials were specifically aimed to establish the following objectives:

Objective (i) To examine whether dose-dependent relationships exists between rates of carbohydrate (sucrose) ingestion and muscle glycogen availability following short-term recovery and/or the capacity for subsequent exercise.

Objective (ii) To explore the potential protein-mediated effects upon muscle glycogen resynthesis and/or the capacity for a subsequent exercise bout with the ingestion of whey protein hydrolysate.

For Objective (i), comparisons were made between a low-carbohydrate ( $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) supplement aimed to restore mostly hepatic glycogen with minimal rates muscle glycogen resynthesis (Casey *et al.*, 2000), and a high-carbohydrate ( $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) supplement designed to elicit high rates of muscle glycogen resynthesis (van Loon *et al.*, 2000b). In regards to Objective (ii), comparisons were made between the same high-carbohydrate supplement stated in Objective (i) to provide carbohydrate in amounts suggested to maximise muscle glycogen repletion during short-term recovery. The energy content of a post-exercise supplement can influence muscle glycogen resynthesis irrespective of macronutrient composition (Roy and Tarnopolsky, 1998). Accordingly, a fraction of the high-carbohydrate supplement was replaced by an isocaloric quantity of protein (i.e. a carbohydrate-protein trial) to explore any protein-mediated effects on muscle glycogen metabolism during recovery and subsequent exercise. Based on the knowledge that further increasing the energy content to  $1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  through the addition of either carbohydrate or protein does not further accelerate muscle glycogen resynthesis relative to when  $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  of carbohydrate is ingested (Beelen *et al.*, 2012; Howarth *et al.*, 2009), a hypercaloric (e.g. providing  $1.2 \text{ g carbohydrate}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  plus  $0.4 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) trial was not examined as treatment arm in **Chapter 5**.

The use of the disaccharide sucrose was chosen on the basis of its potential positive contribution to liver and/or muscle glycogen resynthesis by virtue of equimolar amounts of glucose and fructose. Following exhaustive exercise, sucrose and glucose ingestion seem to elicit similar muscle glycogen resynthesis rates (Blom *et al.*, 1987). However, resting intravenous (Bergstrom and Hultman, 1967b; Nilsson and Hultman, 1974) and oral ingestion (Delarue *et al.*, 1993) studies indicate that fructose preferentially stores liver glycogen relative to glucose, while glucose infusion favours muscle glycogen resynthesis. Given the importance of both liver and muscle glycogen replenishment during short-term recovery and subsequent endurance capacity (Casey *et al.*, 2000), sucrose was deemed a preferable source of carbohydrate to undergo predominant hepatic metabolism (i.e. fructose) to optimise liver glycogen resynthesis alongside a glucose source to maximise muscle glycogen storage (Wallis and Wittekind, 2013).

An important factor determining the rate of muscle glycogen resynthesis is insulin-mediated glucose uptake by the muscle cells (Jentjens and Jeukendrup, 2003). A proposed mechanism for the potential benefit of protein co-ingestion in enhancing the rate of glycogen storage is the synergistic effect of this substrate on insulin secretion (van Loon *et al.*, 2000a; Zawadzki *et al.*, 1992). It has been demonstrated that plasma insulin response increases to a greater extent in whey than in casein protein in its intact form (Reitelseder *et al.*, 2011). The ingestion of a protein hydrolysate accelerates digestion and absorption compared with its intact protein, resulting in a more rapid increase in circulating insulin concentrations (Koopman *et al.*, 2009). This was further confirmed by the finding of greater insulinotropic properties when whey protein hydrolysate was ingested as opposed to whey protein in humans (Morifuji *et al.*, 2010a).

Concerning glycogen storage, the ingestion of whey protein has been shown to stimulate this process more rapidly both in liver and skeletal muscle tissues than when casein was ingested (Morifuji *et al.*, 2005). Furthermore, it appears that ingesting whey protein hydrolysate with carbohydrate augments glycogen resynthesis to a greater extent than when carbohydrate is co-ingested with intact whey protein, casein or intact branched-chain amino acids (Morifuji *et al.*, 2010b). Taken together, these results indicate that a hydrolysed whey protein fraction may have a profound role in

stimulating insulin secretion and concomitant muscle glycogen storage, and thus forming the basis for the inclusion of this protein fraction in **Chapter 5**.

### 3.3 Trial design

**Chapters 4** and **5** included comparisons of two nutritional interventions, with a minimum of two weeks wash-out period being allowed between each main trial to avoid any carry-over effects. The independent variable was the precise nutritional intervention applied during the short-term recovery period. This involves the provision of  $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  and  $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  of carbohydrate in the form of sucrose solutions in **Chapter 4** and a  $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  sucrose *versus* an  $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  sucrose +  $0.4 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  whey protein hydrolysate in **Chapter 5** of the thesis. All treatments were provided in equal volumes ( $10 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) and were matched in sodium and potassium composition. The test beverages were additionally matched in flavour (calorie-free vanilla extract; Flavdrops, Myprotein, UK). Sample analyses of both treatments underwent screening by an independent institution (HFL Sport Science, LGC Ltd., UK) to confirm the absence of any contaminants such as banned anabolic steroids and stimulants including noradrenaline, THG and MDMA. A full description of the treatment composition is provided in Table 3.1.



**Table 3.1** Nutritional composition of the supplements provided in Chapter 4 and 5.

	Low carbohydrate	High carbohydrate	Moderate carbohydrate + protein
<b>Sucrose (g·l)</b>	30	120	80
<b>Lactose (g·l)</b>	–	–	≤3.5*
<b>Protein (g·l)</b>	–	–	40
<b>Fat (g·l)</b>	–	–	≤2.2*
<b>Sodium (g·l)</b>	0.38	0.38	0.38
<b>Potassium (g·l)</b>	0.47	0.47	0.47
<b>Calcium (g·l)</b>	–	–	0.2
<b>Magnesium (g·l)</b>	–	–	0.01
<b>Phosphorous (g·l)</b>	–	–	0.12
<b>Chloride (g·l)</b>	–	–	0.15
<b>Energy (kJ·l)</b>	502	2008	2008

\*= Assay unable to detect values below this number. The caloric content for fat and lactose was therefore assumed negligible.

**Chapter 4** of testing addressed **objective i** by way of a non-randomised repeated measures design. The chosen trial design for **Chapter 4** was based on the premise that a lower rate of sucrose ingestion will mediate sub-optimal recovery and thus resulting in impairment in exercise capacity relative to a higher rate of sucrose intake, as been previously reported in a similar investigation (Betts *et al.*, 2007). It was therefore assumed that participants would run longer when the higher sucrose treatment was administered. Accordingly, a non-randomised design would allow not only an investigation of the metabolic environment at the point of fatigue during the low *versus* high sucrose treatments, but also at the time point in the high sucrose treatment coincident with the onset of fatigue during low sucrose treatment. Indeed, previous studies have adopted a similar approach to investigate mechanisms through which carbohydrate supplementation enhances the capacity for both cycling (Coyle *et al.*, 1986) and running (Tsintzas *et al.*, 1996b) modes of exercise. In addition, participants

were fully familiarised with the trial (see familiarisation section) that were identical to the main procedures and therefore diminishing any order effects. This consequently contributes to higher reliability of the exercise task that would enable the detection of small but worthwhile intervention effects (Hopkins *et al.*, 2001). The second phase (**Chapter 5**) of testing addressed **objective ii** by adopting a randomised double-blind cross-over experimental design.

In **Chapter 6**, the reliability of the employed time to exhaustion measure to assess endurance capacity was assessed using the entire cohort of participants in both **Chapters 4 and 5**.

### 3.4 Participants

The individuals who agreed to participate following the briefing were provided with and informed consent form, indicating their full understanding of the study and their protected rights for confidentiality and withdrawal from the study without giving a reason. Thereafter, a compulsory medical health questionnaire was undertaken by each participant to ensure the absence of any physical, haematologic, metabolic or any other health conditions deemed to pose a risk on the participant or bias towards the investigation. If any of the factors above were present, the volunteer would be deemed unfit to participate in the study and would consequently be excluded from taking part. Further health questionnaires were provided before each trial to ensure that participants were fit and able to take part in testing and to establish similar physical and mental engagements throughout the trials. In relation to eumenorrheic female participants, all measurements were conducted at least three and at most 10 days after the onset of menses (i.e. follicular phase) to ensure low levels of circulating female hormones. Indeed, ovarian hormone changes throughout the menstrual cycle may influence substrate utilisation at rest and during exercise (Campbell *et al.*, 2001), although others have found no differences between menstrual cycle phases (Casazza *et al.*, 2004; Horton *et al.*, 2006). Rather, the influence of ovarian hormones on exercise metabolism may be confounded by other factors that outweigh the effect ovarian hormone variations such as nutritional status (Casazza *et al.*, 2004; Hausswirth and Le Meur, 2011). Thus, while the standardisation of menstrual cycle

phase (follicular phase) was employed in the series of studies in this thesis, this was mainly to increase the likelihood of low concentrations of ovarian hormones, despite that these fluctuation are likely to exert minimal effects of metabolism relative to nutritional status (Campbell *et al.*, 2001).

### 3.5 Anthropometry

The determination of post-void nude body mass for the participants was recorded on their first visit to the laboratory to allow for an accurate nutrient provision during subsequent visits, which were calculated relative to each participant's body mass. Measurements of body mass were also required pre- and post-exercise on all ensuing visits to assess the hydration status of each participant via the recordings of the difference in nude body mass (reported as percentage difference) at the beginning and end of an exercise session. The assumption that any body mass loss is accounted for by loss of body water may not be a direct relationship as a number of potential sources of error such as respiratory water loss, substrate oxidation and metabolic water can influence these calculations (King *et al.*, 2008). Notwithstanding these limitations, body mass loss remains a valuable proxy measure of hypohydration, particularly given the requirement of direct measurement of change in both volume and osmolality of body fluids (Maughan *et al.*, 2007). Weighing of each participant's body mass was undertaken by using a balance scale (Weylux 424, Fereday & Sons Ltd., UK), with a reported capacity of 160 kg accuracy of  $\pm 0.05$  kg. Participants were asked to stand in the centre of the platform while standing aligned with their weight evenly distributed to both feet.

Measurement of the stature of participants was recorded by using a stadiometer (Holtain Ltd., Crosswell, UK) with a reported accuracy of  $\pm 0.1$  cm. The participants were requested to remove any footwear and to hang their arms freely with the palms facing the thighs and the heels. The gluteal area and shoulders were ensured to make contact with the stadiometer to maintain sufficient alignment. To achieve accurate measurements, participants were verbally advised to stand up straight while facing directly with the head in the Frankfort plane (orbitale and tragion are horizontally aligned). Participant were instructed to inspire for measurement, while and the

headboard is lowered to compress the hair (Stewart and Eston, 2007). From the collective measurements obtained above, body mass index was determined ( $\text{kg}\cdot\text{m}^{-2}$ ) for each participant.

### 3.6 Preliminary measurements

Participants were required to visit the laboratories on two occasions to undergo preliminary measurements required for each study. The first visit encompassed the determination of sub-maximal ( $\dot{V}\text{O}_2$ ) and maximal ( $\dot{V}\text{O}_{2\text{max}}$ ) oxygen uptakes. The second visit included familiarisation of the main trials. All exercise tests during the preliminary and main trial phases were running based that were carried out on a motorised treadmill (Ergo ELG70, Woodway, Germany).

#### 3.6.1 Running economy and maximal oxygen uptake

Upon the first visit for each participant, a sub-maximal exercise test was conducted for the estimation of running economy ( $\dot{V}\text{O}_2 \cdot \text{km}^{-1}$ ) and  $\dot{V}\text{O}_{2\text{max}}$ . The protocol commenced with a standardised 5 min warm-up that consisted of jogging at a speed of  $7.5 \text{ km}\cdot\text{h}^{-1}$ . The running economy test was initiated immediately following the warm-up and required participants to run at various sub-maximal speeds with increments of  $1 \text{ km}\cdot\text{h}^{-1}$  that would enable a minimum of four different running speeds (range:  $9\text{-}12 \text{ km}\cdot\text{h}^{-1}$ ). Each running speed consisted of 3 min stages, during which expired gas, heart rate (HR) and ratings of perceived exertion (RPE) were recorded at the final minute of each stage. Expired gas samples, HR and RPE were obtained via Douglas bag method, short range telemetry (Polar FT2, Kempele, Finland) and Borg's (6-20) scale (Borg, 1973), respectively. The data acquired from these tests were subsequently used to calculate the treadmill speeds used during the trial procedures (i.e. speeds that elicit 60, 70 %  $\dot{V}\text{O}_{2\text{max}}$ ) by linear regression (Excel 2010, Microsoft, Redmond, WA, USA).

Approximately 20 min following the sub-maximal test, a run time to volitional exhaustion was included to determine relative  $\dot{V}\text{O}_{2\text{max}}$  values for participants. The

speed for this test was chosen from the data acquired from sub-maximal running that would reflect a speed corresponding to  $\approx 85\%$  of maximum heart rate. This calculated speed was kept constant throughout the test. The initial gradient was set at  $3.5\%$  and was increased with increments of  $2.5\%$  following the end of each running stage. Furthermore, the duration of each running stage (3 min) and collection points (final minute of each stage) of expired air, HR and RPE were kept identical to the sub-maximal testing. The final collection point of data was at the point of volitional exhaustion, which was defined as the final minute to be able to sustain a given running speed indicated by the participant. A final minute expired gas sample was collected at this time point. Satisfactory achievement of  $\dot{V}O_{2\max}$  was fulfilled on the bases of the observation of three of the criteria of the British Association of Sport and Exercise (Bird and Davison, 1997). These included the attainment of a maximal heart rate within 10 bpm from the predicted heart rate maximum for participants ( $220 \text{ beats per minute} - \text{age}$ ), respiratory exchange ratio (RER) values of  $\geq 1.1$  and subjective indication of volitional exhaustion as pointed by the participant on the RPE scale. The data acquired from these tests can then be used to calculate the treadmill speeds that elicit 60 and 70 % of  $\dot{V}O_{2\max}$  required during warm-up and running procedures in the main trials, respectively.

### 3.6.2 Familiarisation

The subjects were required to complete a familiarisation session at least two weeks prior to their main trials. This involved the participants to undergo the identical main exercise protocol procedures (described in experimental protocol section in **Chapters 5 and 6**) but without any collection of venous blood or tissue samples. Participants were only allowed to ingest water during the 4 h recovery period during this visit and fluid provision was in accordance with the main trials (i.e. ingestion of  $10 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  provided in eight aliquots and consumed at 30 min intervals). A main purpose of this visit is to familiarise the participants to the experimental procedures and apparatus and thereby diminish any learning and/or trial effects. The test was also aimed to confirm the estimated relative speeds that corresponded to the required intensity (i.e. 60 % and 70 %  $\dot{V}O_{2\max}$ ) during the trials, with any adjustments to speed deemed by expired gas data being applied accordingly.

### 3.7 Physiological measurements

#### 3.7.1 Urine output

Baseline urine samples were obtained 30 min before testing to determine hydration via freezing point depression method by using a cryoscopic osmometer (Advanced Instruments, Inc., USA). The threshold for adequate hydration was assumed for osmolality values  $\leq 900 \text{ mOsm} \cdot \text{kg}^{-1}$  (Shirreffs and Maughan, 1998). During the 4 h recovery period, the voided urine was collected in a scaled vessel for the quantification of total urine output during the entire 4 h recovery period.

In **Chapter 5**, during the 4 h recovery period, the voided urine was collected in a vessel containing a preservative (5 ml of 10 % thymol-isopropanol). Once total urine output during this period was recorded, a mixed 1 ml sample was taken and stored at  $-80^\circ\text{C}$ . The urea concentration of this sample (according to procedures identical to those applied during plasma urea analysis) was then used as an estimate of total urine nitrogen excretions. Plasma urea concentrations were subsequently used to correct urinary urea excretion for whole-body determination of urea pool during recovery (Livesey and Elia, 1988). Non-protein respiratory exchange ratio (NPRER) was calculated to reflect protein oxidation rates during the 4 h recovery phase (Jequier *et al.*, 1987).

#### 3.7.2 Expired gas sampling

Expired gas samples were obtained via Douglas bag method (Hans Rudolph, MO, USA) from each participant. A respiratory valve with a mouth piece was attached to a 200 L Douglas bag for expired gas sampling, which was provided with a nose clip to participants approximately 30 seconds before sampling to remove any residual atmospheric air from the valves. The collected gas samples were then analysed for relative expired fractions of oxygen and carbon dioxide using a paramagnetic and infra-red analysers, respectively (Servomex, Crowborough, UK). The total volume of expired gas within the Douglas bag was subsequently measured by a dry gas meter

(Harvard Apparatus, UK), with the temperature of expired gases being collected at the time of evacuation by a thermistor probe (Grant Instruments Ltd., Cambridgeshire, UK). Prior to any gas collection, calibration of equipment was conducted using gas cylinders containing specific atmospheric gases with a known relative composition ( $N_2 = 0\%$ ;  $O_2 = 16.9\%$ ;  $CO_2 = 4.93\%$ ), as validated by the manufacturer (CryoService, UK). Each analyser was then validated against atmospheric air. The inspired air was measured proximally to the participants throughout the studies, with values used relative to each corresponding expired gas sample to minimise systematic bias associated with assumptions that inspired gas fractions are stable and reflective of atmospheric constants (Betts and Thompson, 2012).

The calculations of oxygen consumption and carbon dioxide production from each bag were then used for the determination of whole-body carbohydrate and lipid oxidation rates ( $g \cdot min^{-1}$ ) using stoichiometric formula (Jeukendrup and Wallis, 2005), assuming that participants were exercising at moderate to high intensity (50-75 %  $\dot{V}O_{2max}$ ):

$$\text{Carbohydrate Oxidation} = (4.210 \cdot \dot{V}CO_2) - (2.962 \cdot \dot{V}O_2)$$

$$\text{Fatty Acid Oxidation} = (1.695 \cdot \dot{V}O_2) - (1.701 \cdot \dot{V}CO_2)$$

The calculations were based on the assumption of negligible protein oxidation during prolonged exercise. Indeed, incorporating isotopic tracers ( $L-[^2H_5]$  phenylalanine model), which take into account whole-body protein synthesis, breakdown and oxidation, indicate that the rates of protein oxidation during 4 h post-exercise recovery with carbohydrate supplementation reach  $\approx 1 g \cdot h^{-1}$  measured following 6 h of prolonged exercise (Koopman *et al.*, 2004). Thus, in the absence of protein supplementation in **Chapter 4**, the assumption of negligible protein oxidation during recovery should not influence the calculations.

Ingesting  $\approx 20 g \cdot h^{-1}$  protein prior to and during 6 h of prolonged exercise in addition to 4 h of recovery has been shown to increase protein oxidation to  $\approx 1.94 g \cdot h^{-1}$  during post-exercise recovery (Koopman *et al.*, 2004). In accordance, the calculations of

substrate metabolism ( $\text{g} \cdot \text{min}^{-1}$ ) in **Chapter 5** included estimation of protein oxidation using the following stoichiometric formula, assuming that participants were exercising at moderate to high intensity (50-75 %  $\dot{V}\text{O}_{2\text{max}}$ ):

$$\text{Carbohydrate Oxidation} = (4.212 \times \dot{V}\text{CO}_2) - (3.005 \times \dot{V}\text{O}_2) - (2.449 \times \text{N})$$

$$\text{Fatty Acid Oxidation} = (1.754 \times \dot{V}\text{O}_2) - (1.754 \times \dot{V}\text{CO}_2) - (2.017 \times \text{N})$$

Where N is the estimated rate of nitrogen excretion based on urinary/plasma urea (Livesey and Elia, 1988).

Extra-muscular carbohydrate oxidation from both experiments was then derived from the difference between whole-body carbohydrate oxidation as determined from indirect calorimetry and intramuscular carbohydrate oxidation methods (overall muscle glycogen degradation).

### 3.7.3 Blood sampling

Following the application of a topical anaesthetic (4 % Ametop gel, Smith & Nephew, Hull, UK), venous blood samples were obtained during the main trials through an indwelling cannula (BD Venflon pro, Oxford, UK) inserted into an antecubital vein. The cannula was kept patent throughout the trials by flushing with heparin-free isotonic saline (B. Braun; Melsungen, Germany) following each sampling point. Participants remained rested in a semi-supine position for 10 min prior to any baseline blood sample. A 10 ml blood sample was collected during each sampling point before being dispensed into 2 x 5 ml tubes. Each blood sample was firstly transferred into a non-anticoagulant collection tube (Sarstedt, Leics, UK), and left to clot for  $\approx 45$  min at room temperature before centrifugation at 2000 xg for 10 minutes at 4°C (Heraeus Primo R; Thermo Fisher Scientific, Loughborough, UK) for serum extraction, which was stored at -80°C pending analysis for insulin concentration. The remaining 5 ml of each venous blood sample was dispensed into a different tube containing an anti-coagulant (ethylenediaminetetraacetic acid; EDTA) then immediately analysed for haemoglobin concentrations by using an automated haematology analyser (Sysmex SF-3000; Sysmex Ltd., UK). Thereafter, 3 x 50  $\mu\text{l}$  aliquots of blood were removed



using micro-haematocrit tubes and subsequently centrifuged (Hawksley, UK) to obtain haematocrit. Equations based on haemoglobin and haematocrit values were utilised to determine plasma volume changes throughout the trials (Dill and Costill, 1974). The remaining EDTA-treated blood was then spun for centrifugation under 2000 xg for 10 minutes at 4°C for plasma extraction prior to being stored at -80°C for later analysis of plasma glucose, lactate, non-esterified fatty acids and urea.

### **3.7.4 Blood analysis**

#### **3.7.4.1 Plasma glucose, non-esterified fatty acids, lactate, and urea**

An automated spectrophotometric analyser (RX Daytona, Randox, Crumlin, Ireland) was utilised for the assessment of plasma glucose, non-esterified fatty acids, lactate and urea. A calibration was conducted prior to each sample analysis and quality control was checked against manufacturer available standards.

#### **3.7.4.2 Serum insulin**

Sera concentrations were assessed by enzyme-linked immunosorbent assays (ELISA; Mercodia, Uppsala, Sweden) using a spectrophotometric plate reader (Spectrostar Nano, BMG Labtech, Ortenberg, Germany). The fundamental principle of all these assays is the target analyte (the antigen) is recognised with high specificity by antibodies within the human body. The immune system produces antibodies in response to the presence of antigens. These antibodies subsequently recognise and bind to the antigens, and the labelling of resultant bound antibody forms the basis of this method (Crowther, 2001).

## **3.8 Muscle biopsy procedures**

### **3.8.1 Muscle biopsy sampling**

A needle biopsy technique (Bergstrom, 1962) was used to obtain muscle tissue from the *vastus lateralis* throughout the series of experiments. This specific site was chosen to present muscle glycogen data that are comparable with the majority of

investigations conducted in this area; the large contribution of the quadriceps muscles during treadmill running and; the *vastus lateralis* constitutes the largest volume ( $\approx 35\%$ ) of total *quadriceps femoris* (Barnouin *et al.*, 2014).

Concerning the mode of exercise, it has been demonstrated that muscle glycogen utilisation (obtained from the *vastus lateralis*) is greater in cycling than running during 60 min of exercise at  $\approx 74\% \dot{V}O_{2\max}$  (Arkinstall *et al.*, 2001). However, this may be related to the differences in the metabolic demands as a result of the use of different muscle groups (Costill *et al.*, 1974). Cycling exercise involves a substantial contribution of the *vastus lateralis*, such that almost full depletion of muscle glycogen in the *vastus lateralis* is commonly reported following prolonged exhaustive cycling (Hermansen *et al.*, 1967). While earlier evidence suggested that long-distance running results in an equal contribution of the lateral aspect of the *quadriceps femoris*, *gastrocnemius* and lateral aspect of the *gluteus* muscles (Karlsson and Saltin, 1971), follow-up evidence reports greater pre-exercise glycogen storage and subsequent utilisation by the *soleus* muscle relative to both the *gastrocnemius* and *vastus lateralis* during running. This was further supported by another study (Krssak *et al.*, 2000) whereby glycogen utilisation in the *triceps surae* was greater than the *vastus lateralis* during treadmill running. Therefore, from the aforementioned studies it can be postulated that a greater depletion rate (and hence resynthesis due to the intimate relationship between the magnitude of muscle glycogen depletion and its rate of resynthesis; (Zachwieja *et al.*, 1991)) may be present in the *triceps surae* than the *vastus lateralis*. Nevertheless, prolonged treadmill running has been shown to substantially deplete muscle glycogen to critically low levels in the *vastus lateralis* (Tsintzas *et al.*, 1996a). Thus, measurements obtained from the *vastus lateralis* were assumed to reflect total contribution of muscle glycogen towards overall metabolism under the assumption that dry muscle mass of both legs was 6 % of body mass and that glycogen utilisation of these limbs is represented by the *vastus lateralis* sample.

The skin around the sampling site was shaved if necessary and was then sterilised by using iodine solution while participants were in a semi-supine position. Following a small incision ( $\approx 2\text{--}3\text{ mm}$ ) in the skin and fascia using a scalpel blade and performed

under a local anaesthetic (1 % Lidocaine; Hameln Pharmaceuticals Ltd., UK), a 5 mm gauge Bergstrom biopsy needle (Roberts Surgical Healthcare Ltd., UK) was used to obtain 30-100 mg of wet muscle tissue. The biopsy samples were taken in a step-wise manner such that each biopsy was taken proximally to any preceding sampling site and a distance of at least 2.5 cm was allowed between each prospective site to minimise the effects of an inflammatory response on muscle metabolite concentrations (Van Thienen *et al.*, 2014). An opposite leg was used during the second main trial for each participant, where the use of dominant/non-dominant limbs was counterbalanced between subjects.

### 3.8.2 Muscle biopsy analysis

Once removed from the leg, the muscle tissue was extracted immediately from the needle biopsy and was snap-frozen into liquid nitrogen that was dispensed into a metal kidney dish. The muscle samples were then removed from the kidney dish, immediately placed into a ventilated cryogenic vial (Corning, Ewloe, UK) and transferred into a larger liquid nitrogen canister where the specimens were stored for later analysis. Upon removal from storage but while still immersed in liquid nitrogen, each sample was dissected to remove 15-30 mg fragment of muscle and subsequently placed in a freeze-dryer (Modulyo, Edwards, UK) for  $\approx 18$  h at  $-50^{\circ}\text{C}$  and between  $10^{-1}$  and  $10^{-2}$  mbar. Thereafter, the muscle powdering procedure took place at room temperature using a surgical blade, agate pestle and mortar to remove all visible blood and connective tissue and reduce each muscle specimen to fine powder. The resultant powder was divided equally between 2 Eppendorf tubes and the precise weight of each sample was determined by using an electrical balance scale (Mettler AE240, Switzerland) before being stored with silica gel at  $-80^{\circ}\text{C}$  for later analysis for total for phosphocreatine (PCr), creatine (Cr) and muscle glycogen concentrations. The relative concentrations of these metabolites were determined according to enzymatic methods previously described (Harris *et al.*, 1974; Lowry and Passonneau, 1972) using a spectrophotometric plate reader (SpectraMax 190, Molecular Devices, USA). The principle of these methods are based on enzyme catalysed reactions with the coenzymes  $\text{NAD}^{+}$  and  $\text{NADP}^{+}$  being simultaneously reduced to NADH and NADPH, respectively (or *vice versa* for muscle creatine concentration). Thus, using the coefficient of extinction of NADH and NADPH, metabolite concentrations were

calculated from the change in sample absorbency in relation to double-distilled water blanks using the equations in Appendix E.

To account for any contamination of the obtained muscle specimens with blood, lipid or connective tissue, the concentration of total muscle glycogen was normalized in relation to each participant's total creatine (PCr+Cr) concentration across both trials (i.e. the average total creatine concentration for all biopsies from each participant). Glycogen was assayed by hydrolysis in 1 mol/l hydrochloric acid (HCl). Acid hydrolysis of the insoluble fraction of glycogen required the addition of 0.1 ml of HCl per mg of muscle powder to the precipitated muscle pellet, while acid hydrolysis of the supernatant (extract) required the addition of 0.1 ml of HCl to the 20  $\mu$ l of undiluted neutralised extract. These mixtures were incubated for 2 h in tightly screwed Eppendorf tubes at 100°C in a dry bath (Grant BTS, UK) before centrifugation for 1 min at 1400 xg. Thereafter, 15  $\mu$ l of sodium hydroxide (NaOH) were added to neutralise the acid hydrolysed extract. The hydrolysed extracts and pellets were diluted as necessary and total mixed-muscle glycogen concentration was calculated by adding the acid-soluble and acid-insoluble glycogen concentrations (Jansson, 1981). Moreover, the glucose concentration in the condensed neutralised extract was also determined to enable the acid-soluble glycogen fraction to be corrected for any free glucose within the muscle. Total muscle glycogen concentrations are reported as mmol glucosyl units per kilogram of dry mass ( $\text{mmol}\cdot\text{kg dm}^{-1}$ ) to account for any measurement error associated with fluid shift during exercise. The contribution of muscle glycogen towards whole-body carbohydrate oxidation during Run-2 was estimated from lean tissue mass of all leg muscle (6 % of body mass) from a typical 72.1 kg trained individual using dual-energy X-ray absorptiometry (DXA) analysis (Betts *et al.*, 2008). The procedures for acid hydrolysis of the muscle sample, measurement of PCr, Cr, acid-soluble and acid-insoluble glycogen concentrations are detailed in Appendix E.

### **3.8.3 Decontamination procedures**

Once the wet muscle tissue extraction process has been completed, all equipment used in muscle biopsy technique underwent a generic decontamination procedure. All

muscle biopsy component parts were disassembled and were cleaned individually with warm water. A wire brush was then used to remove any organic matter, blood or any other deposits prior to placement in an Ultrasonic bath containing a solution of 0.3 % Sonozyme enzymatic soap for sonification for 10 min at 30 °C. Once sonified, all equipment were rinsed with ultrapure water before they were immersed under a disinfectant (Milton Sterilising Tablets, UK) for 15 min before they underwent another rinse cycle with ultrapure water. Finally, each item was individually placed in a sterilising pouch prior to an autoclave at 121 °C for 20 min.

### 3.9 Subjective Measurements

Subjective ratings of perceived exertion (RPE) were obtained during all exercise trials by using Borg's 6-20 RPE scale (Borg, 1973), where 6 and 20 range from 'very very light' to 'maximum' effort. Subjective measurements of stomach discomfort, gut fullness and thirst were recorded using adapted Borg scales whereby scales ranged from 'no discomfort' to 'extreme discomfort', 'not full' to 'very very full' and 'not thirsty' to 'very very thirsty', respectively. To account for any mood disturbances during any experiments, a profile of mood state (POMS) questionnaire was completed by each participant prior to commencing any of the trials. This questionnaire was shown to be a viable tool to detect any mood fluctuations in an exercise setting that may have an influence on performance (Beedie *et al.*, 2000; Berger and Motl, 2000). Participants completed a 37-item short form profile of mood state (POMS-SF) questionnaire (Shacham, 1983). Participants indicate the degree to which each of the 37 items describes their feelings by using a 5-point Likert scale format where 0 indicates "not at all" and 4 "extremely". POMS-SF items are divided into six categories: tension, depression, anger, fatigue, confusion and vigour. Total mood disturbance (TDM) was then calculated as the sum of the first five categories minus vigour.

### **3.10 Control measures**

#### **3.10.1 Standardisation of lifestyle**

Over the 48 h prior to the familiarisation trial, a weighed dietary record was completed by each participant for the analysis of macronutrient composition and total daily energy intake by using a nutritional software package (Nutritics, Dublin, Ireland). The same diet was then replicated prior to any of main trial. Participants were required to abstain from alcohol consumption 24 h before any trial, while caffeine abstinence employed at 17:00 on the day preceding any trial. The latter aimed mainly to avoid any unnecessary side effects that may negatively influence performance as a result of adverse withdrawal from caffeine (Jeacocke and Burke, 2010). Approximately 12 h before the familiarisation session and subsequently before the experimental trials, a standardised meal was provided for each participant. This was aimed to minimise any within subject variability in the nutritional status of each participant that is known to influence metabolism and exercise performance and ultimately may influence the outcomes of the study (Braun and Brooks, 2008; Jeacocke and Burke, 2010).

Participants were required to complete an activity log in conjunction with their dietary control procedures discussed in the experimental protocol. The participants were instructed to refrain from any strenuous physical training 48 h before any trial with any light-to moderate habitual training recorded for time, duration and mode of exercise before the familiarisation session. This procedure was matched for ensuing trials. Standardisation of lifestyle was retrospectively analysed for nutritional intake (via nutritional assessment software) and activity (minutes of exercise per day) to ensure sufficient control over the testing period.

#### **3.10.2 Environmental measurements**

Ambient temperature, humidity and barometric pressure was monitored and recorded at 60 minute intervals throughout the trials using a portable weather station (WS 6730; Technoline, Germany). The latter was then used to record atmospheric pressure to allow for corrections to standard volumes during expired gas analysis.

### 3.11 Reliability measurements

Where applicable (i.e. blood metabolites and muscle glycogen), samples from each participant were assayed in the same plate to minimise inter-assay variation. To evaluate the error variation and establish sufficient precision of measurement, coefficients of variation ( $CV \% = SD/mean \times 100$ ) were assessed for intra-assay and accompanying measures obtained during the current series of studies. The CV % for each analyte measured throughout the experimental chapters in this thesis are presented in Table 3.2, with the results being obtained from seven separate analyses of each sample. Due to the limitations to muscle tissue availability, the CV % for muscle glycogen is presented as the mean of variance across duplicate absorbencies.

**Table 3.2** Coefficient of variation of different parameters measured throughout the experimental chapters

parameter	Method	CV %
Glucose	Spectrophotometry	1.1
Non-esterified fatty acids	Spectrophotometry	2.5
Lactate	Spectrophotometry	1.0
Urea	Spectrophotometry	1.7
Haemoglobin	Spectrophotometry	0.4
Albumin	Spectrophotometry	1.3
Insulin	Spectrophotometry	3.4
Urine osmolality	Osmometry	0.4
Total muscle glycogen	Spectrophotometry	2.0

### 3.12 Statistical analysis

Any data that require a single comparison between two means was tested for normality of distribution using a Shapiro-Wilk test. A paired sample *t*-test was performed to those data deemed normally distributed, while a non-parametric

equivalent (i.e. Wilcoxon test) was used to compare data deemed in violation of normality. Where multiple comparisons were made to assess the effects treatment over time, a two-way linear mixed model was employed, with trial and time inserted as covariates and participants inserted as random effects. This statistical approach allows to model for nonlinear changes in a dependent variable across time, which is often associated with a number of physiological variables (e.g. blood metabolites and heart rate), while also acknowledging both group and individual changes over time (Krueger and Tian, 2004). Where a significant interaction effect was identified, multiple *t*-tests were performed to compare the means of interest and a Bonferroni step-wise correction was employed to determine the location of the variance (Atkinson, 2002b).

A *priori* sample size estimates were conducted to determine the number of participants required to provide a threshold of at least 80 % power to detect an intervention effect. While the primary outcome measure in these experiments is the rate of muscle glycogen resynthesis/degradation, this variable is highly reliable relative to other outcomes and, accordingly, this consistency results in sample size estimates requiring only 4 or 5 participants (thus justifying the use of typically 6 or 8 in most studies in this field). It was therefore deemed appropriate to base sample size estimates on secondary less reliable outcome measures. In this case, exercise capacity exhibits the highest between subject variability and previous work that contributes directly to the rationale for this study observed a difference between the high sucrose and moderate sucrose plus whey protein supplements under investigation here of 7.5 min, with a standard deviation of differences of 5 min (Betts *et al.*, 2007). Based upon these data it can be estimated that a sample size of 6 would be appropriate to provide a 85 % power to detect such effects even on these more variable secondary outcomes using a two tailed paired *t*-test. Statistical procedures were performed using commercially available software (IBM SPSS version 20.0, SPSS Inc., Chicago, IL) and significance is set at an alpha level of 0.05. All results are reported as the mean  $\pm$  standard deviation (SD) of the mean unless stated otherwise.

Unless explicitly reported, the error bars displayed on figures are confidence intervals (CI) that have been corrected for between-subject variance (Franz and Loftus, 2012). The normalised CI are based on an equation (Appendix Hi) that removes between



subject differences and allow traditional SEM computations that were involved during statistical testing. The magnitude of these CI directly infers differences between the observed means (i.e. statistical significance) and not individual variance around the mean. In **Chapters 4** and **5**, the incremental area under the concentration curve (iAUC) for plasma glucose and serum insulin during the 4 h recovery period were calculated (Appendix Hii) by incorporating the method recommended by Wolever (Wolever, 2004).

## CHAPTER 4

### IMPACT OF MUSCLE GLYCOGEN AVAILABILITY ON THE CAPACITY FOR REPEATED EXERCISE IN MAN

#### Introduction

Endurance capacity during an initial prolonged exercise bout is primarily reliant upon pre-exercise glycogen availability, such that muscle glycogen content exhibits a direct positive correlation with exercise time to exhaustion (Bergstrom *et al.*, 1967; Tsintzas *et al.*, 1996a; Tsintzas *et al.*, 2001). Similarly, muscle glycogen repletion can impact the time required to recover functional capacity, with carbohydrate intake accelerating both glycogen resynthesis and restoration of exercise capacity relative to when no carbohydrate is ingested (Fallowfield *et al.*, 1995; Gejl *et al.*, 2014; Jentjens and Jeukendrup, 2003). Furthermore, carbohydrate ingestion rate exhibits a dose-dependent relationship with the rate of muscle glycogen resynthesis until a threshold of ingesting  $\approx 1.2 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$  (Betts and Williams, 2010). It is therefore logical to postulate that increasing carbohydrate intakes might also exhibit a dose-dependent relationship with the restoration of exercise capacity following short-term recovery. However, while some information is available pertaining to muscle glycogen metabolism during a subsequent exercise bout (Berardi *et al.*, 2006; Betts *et al.*, 2008; Tsintzas *et al.*, 2003), it remains merely an assumption that muscle glycogen availability is an important determinant of fatigue during a second bout of exercise following short-term recovery.

Based on the few studies to have examined the relationship between carbohydrate ingestion rate in recovery and restoration of exercise capacity, most report no consistent pattern (Betts *et al.*, 2007; Fallowfield and Williams, 1997; Wong and Williams, 2000), with only one reporting a dose-dependent relationship (Betts *et al.*, 2007). Notwithstanding that the aforementioned studies did not provide any glycogen data, there is some evidence that glycogen resynthesis (in particular liver glycogen) is an important determinant of endurance capacity following short-term recovery (Casey

*et al.*, 2000). This is understandable given that liver glycogen content is preferentially resynthesised over muscle glycogen when modest amounts of carbohydrate ( $\approx 0.3 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) are ingested following an initial exhaustive exercise bout (Casey *et al.*, 2000). Conversely, the capacity for repeated exercise has also been dissociated from skeletal muscle glycogen availability in other studies (Berardi *et al.*, 2006; Betts *et al.*, 2007; Betts *et al.*, 2008). It is therefore possible that fatigue during repeated exercise may manifest differently from an initial prolonged exercise bout, and the availability of muscle glycogen may not be the primary cause of fatigue during subsequent exercise under all conditions. Accordingly, there is an outstanding need for improved understanding about the relative importance of muscle glycogen availability in offsetting fatigue during a repeated exercise bout as opposed to an initial bout, with implications for the optimal carbohydrate feeding strategy in recovery to maximise not only glycogen resynthesis but also restoration of exercise capacity.

To this end, the current study nutritionally manipulated carbohydrate availability during short-term recovery to examine metabolic and ergogenic outcomes during subsequent exercise. Specifically, we sought to examine whether muscle glycogen availability is associated with fatigue in a repeated exercise bout following short-term recovery. Comparisons were therefore made between a low-carbohydrate (L-CHO) supplement sufficient only to restore hepatic glycogen with minimal rates of muscle glycogen resynthesis (Casey *et al.*, 2000), and a high-carbohydrate (H-CHO) supplement designed to elicit high rates of muscle glycogen resynthesis (van Loon *et al.*, 2000b). It was hypothesised that the extended run time to fatigue expected with increasing carbohydrate intake would be explained by a proportional acceleration of muscle glycogen resynthesis during recovery and thus greater glycogen availability during repeated exercise.

## Materials and Methods

### Participants

Nine healthy recreationally active men and one woman participated in the study. The characteristics of this sample were: age  $21 \pm 1$  years; body mass (BM)  $72.5 \pm 8.2$  kg; height  $180 \pm 9$  cm;  $\dot{V}O_{2\max}$   $61 \pm 6$  ml·kg<sup>-1</sup>·min<sup>-1</sup>; weekly exercise duration  $5 \pm 3$  h. The participants were informed about the possible risks and discomforts involved before giving their voluntary consent to take part. The current study has been approved by the local National Health Services Research Ethics Committee (Ref: 09/H0101/82) with a controlled clinical trial number: ISRCTN87937960.

### Experimental design

Each participant performed two main trials in a repeated measures experimental design interspersed by an interval of  $\geq 2$  weeks. A weighed dietary record was completed 48 h preceding the familiarisation trial, and was subsequently repeated prior to the commencement of the main trials ( $11037 \pm 2963$  kJ·d<sup>-1</sup>;  $55 \pm 5$  % carbohydrate;  $17 \pm 3$  % fat;  $28 \pm 4$  % protein). Participants were provided with a standardised meal (3180 kJ; 57 % carbohydrate; 24 % Protein; 19 % fat) in the evening ( $12 \pm 1$  h) before the familiarisation trial and replicated this prior to each main trial. Participants were also requested to abstain from alcohol consumption and refrain from strenuous physical activity for 48 h pre-trial, with any light exercise recorded and matched during the period of standardisation of lifestyle for ensuing trials.

The main trials required participants to run to the point of volitional exhaustion (Run-1) at an intensity of 70 %  $\dot{V}O_{2\max}$  followed by a 4 h recovery period, where participants ingested a low carbohydrate (L-CHO) or a high carbohydrate (H-CHO) supplement. Following recovery, a second run to exhaustion (Run-2) at the same exercise intensity (i.e. 70 %  $\dot{V}O_{2\max}$ ) was undertaken by each participant to assess restoration of exercise capacity. As has been successfully applied in previous studies that have contrasted relative and absolute fatigue points to understand fatigue

mechanisms in relation to running (Tsintzas *et al.*, 1996a) and cycling (Coyle *et al.*, 1986), trial order required L-CHO to be completed first. Previous data (Betts *et al.*, 2007) has reported that the restoration of exercise capacity can be dose-dependent with ingestion of moderate-high *versus* high carbohydrate during short-term recovery. Differences in exercise time to exhaustion can therefore be confidently expected between the more markedly different very low *versus* high carbohydrate doses in this study. Accordingly, establishing the absolute time-point of fatigue in L-CHO trials prior to H-CHO trials enables comparisons in the metabolic environment both at the point of volitional fatigue in both treatments and at the time point in the H-CHO treatment that corresponds to fatigue during L-CHO treatment.

Consistent with the above rationale, muscle biopsy samples were obtained in L-CHO trial: upon cessation of Run-1; post-recovery; and volitional exhaustion during Run-2 (F2). In the H-CHO trial, the three muscle biopsy samples were obtained: post-recovery; the time point coincident with fatigue in L-CHO (F2); and the point of volitional exhaustion during the subsequent exercise bout (F3). As a result of the dietary and activity standardisation, and the fact the participants ran to the point of volitional exhaustion, negligible intra-individual variability in muscle glycogen levels following Run-1 were expected between trials, as previously reported in a similar protocol (Tsintzas *et al.*, 1996a) and this was further verified by the well-matched times to exhaustion during Run-1 in both trials (results section). Thus, the sample obtained following Run-1 in L-CHO merely serves to verify the expected substantial glycogen depletion from the exercise protocol, whilst the remaining samples across both trials inform the primary research questions pertaining to differences in muscle glycogen availability immediately prior to and during the second exercise bout.

### **Experimental protocol**

The experimental protocol pertaining to the current study is described in further detail elsewhere (Alghannam *et al.*, 2014). Each participant arrived to the laboratory at 08:00  $\pm$  1 h following an overnight fast ( $\geq$  10 h). Upon arrival at the laboratory, participants completed a profile of mood state (POMS) questionnaire, before a baseline urine sample was obtained. Post-void nude body mass (BM) was then

recorded (Weylux 424, UK) before a 5 min resting expired gas sample was collected using the Douglas bag technique. An indwelling cannula was inserted into an antecubital vein and a 10 ml baseline venous blood sample was collected. Participants commenced the exercise protocol with a standardised 5 min warm-up at 60 %  $\dot{V}O_{2\max}$ , where speed was then increased to 70 %  $\dot{V}O_{2\max}$  until the point of volitional exhaustion ( $11 \pm 1 \text{ km}\cdot\text{h}^{-1}$ ). During Run-1, one minute expired gas samples, heart rate (HR; Polar FT2, Kempele, Finland), ratings of perceived exertion (RPE), and 10 ml blood samples were collected (Figure 4.1). Water intake was permitted *ad libitum* during the familiarisation trial ( $0.5 \pm 0.3 \text{ L}$  during Run-1) and then matched for subsequent trials. To accurately gauge relative levels of fatigue, participants were permitted to reduce the intensity (walking at  $4.4 \text{ km}\cdot\text{h}^{-1}$ ) for 2 min intervals on two occasions when they indicated that they could not maintain the prescribed intensity, followed by a return to the treadmill speed equivalent to 70 %  $\dot{V}O_{2\max}$ . Only on the third occasion that participants indicated that they were unable to run at the prescribed speed was volitional exhaustion accepted. Immediately following Run-1 in L-CHO trial, participants rested on an adjacent bed in a semi-supine position while  $\approx 80 \text{ mg}$  of muscle was obtained from the *vastus lateralis* by percutaneous needle biopsy technique (Bergstrom, 1962) from a 3-5 mm incision made prior to exercise at the anterior aspect of the thigh using a surgical blade under local anaesthetic (1 % lidocaine; Hameln Pharmaceuticals Ltd., Brockworth, UK). Thereafter, the first bolus of prescribed solution was immediately provided and recovery time commenced, before nude BM mass was recorded to assess hydration status through percentage change in mass (with body mass adjusted for the ingested bolus). The remaining seven aliquots of prescribed solution were provided at 30 min intervals (Figure 4.1). Participants were permitted 15 min to consume each volume, and subjective measures of stomach discomfort, gut fullness and thirst were recorded following the ingestion of each bolus using adapted Borg scales (Alghannam *et al.*, 2014). Expired gas followed by venous blood samples were collected hourly prior to fluid provision. Furthermore, urine output was collected throughout the 4 h recovery period. Approximately 3 h 40 min into recovery, two (in L-CHO trial) or three (in H-CHO trial) 3-5 mm incisions were made proximally on the same leg at least 3 cm apart followed by obtaining a muscle biopsy sample at the end of recovery (with the remaining incisions dressed for easy access at later sampling points), with the order of

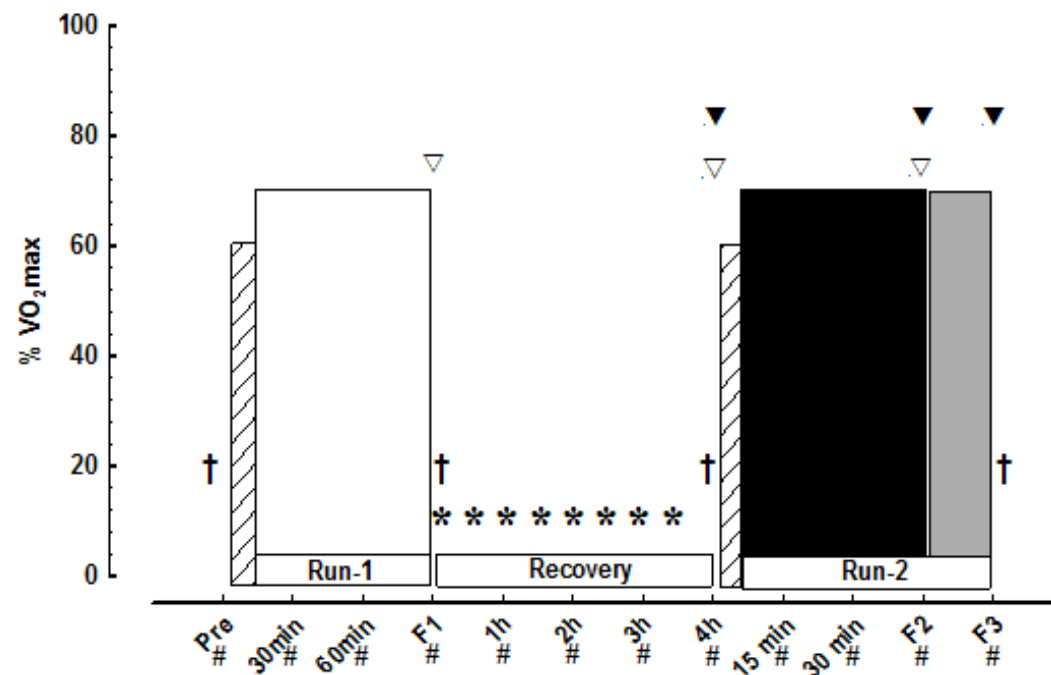
dominant/non-dominant legs for muscle biopsy sampling being counterbalanced between the main trials. Nude BM was recorded at the end of recovery, and participants initiated a standardised warm-up before running at 70 %  $\dot{V}O_{2\max}$  to volitional exhaustion. As for Run-1, water intake was permitted *ad libitum* during the familiarisation trial and matched for subsequent trials ( $0.3 \pm 0.3$  L during Run-2). Reaching the point of volitional exhaustion was determined in an identical manner to the initial exercise bout. Expired gases, HR, RPE and venous blood samples were also collected during Run-2 (Figure 4.1). In the L-CHO trial fatigue was reached after  $48 \pm 11$  min, at which point the one remaining incision site in that trial was used to obtain a final muscle biopsy. Therefore, after  $48 \pm 11$  min in the subsequent H-CHO trial, the exercise protocol was briefly ( $624 \pm 236$  seconds) interrupted to obtain a muscle biopsy at the time point coincident with fatigue during L-CHO trial (i.e. F2) – thus permitting comparison of glycogen concentrations at a matched absolute time-point and depletion over a matched period between the two nutritional interventions, as employed previously (Tsintzas *et al.*, 1996a). Participants then mounted the treadmill and continued to run until volitional exhaustion before the final biopsy (i.e. F3) was obtained from the remaining incision site. BM was subsequently recorded following the attainment of the final biopsy from each participant. Ambient temperature and humidity were recorded at 60 min intervals throughout the trials using a portable weather station (WS 6730; Technoline, Berlin, Germany) and were not different among the trials:  $20.3 \pm 0.5$  and  $20.1 \pm 0.5^\circ\text{C}$ ; and  $46 \pm 2$  and  $47 \pm 2$  % in L-CHO and H-CHO trials, respectively. Background music was standardised between trials and participants were unaware of the time elapsed during the exercise capacity test, with all verbal encouragement standardised.

### **Solution composition**

The rates of carbohydrate (sucrose) intake in the L-CHO and H-CHO trials were  $0.3 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  and  $1.2 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$ , equating to a total amount of carbohydrate provided during the recovery period of  $87 \pm 10$  g and  $349 \pm 41$  g in L-CHO and H-CHO beverages, respectively. All treatment solutions were isovolumetric ( $10 \text{ ml}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$ ) relative to each participant's BM ( $727 \pm 86 \text{ ml}\cdot\text{h}^{-1}$ ), thus formulating a 3 % and 12 % solution in L-CHO and H-CHO respectively. Both supplements were matched for their electrolyte content (sodium and potassium) and were flavour

matched. Full information pertaining to the nutritional treatments is provided elsewhere (Alghannam *et al.*, 2014). Owing to the design of the experiment (i.e. participants were aware of the number of biopsies planned during each run), the treatments were not blinded.





**Figure 4.1** A schematic representation of the study protocol. †, body mass assessment; \*, fluid provision; #, expired gas and blood sample; ∇, muscle biopsy during L-CHO; ▼, muscle biopsy during H-CHO; F1, fatigue in Run-1; F2, fatigue in L-CHO; F3, fatigue in H-CHO; dashed columns, warm-up; black column, run time to exhaustion in L-CHO trial; grey column, extended run time to exhaustion with H-CHO treatment during Run-2.

### Statistical analysis

*A priori* sample size estimation was conducted based on the exercise capacity data of a similar previous study (Betts *et al.*, 2007) which showed that a sample size of  $n=6$  would provide 90 % power to detect a difference in exercise capacity of 16.2 min using a two-tailed *t*-test between two carbohydrate supplements with differing amounts. Paired *t*-tests were used to analyse data involving a single comparison of two level means. Where paired-difference data were deemed non-normally distributed by Shapiro-Wilk test, values are reported as median (range), with Wilcoxon signed rank test being used to compare medians. A two-way linear mixed model with repeated measures (time x trial) was employed to identify overall differences between experimental conditions. Wherever a significant interaction effect was found, a Bonferroni step-wise correction was employed to determine the location of the variance (Atkinson, 2002b). Pearson product moment correlation coefficient (*r*) was used to explore the relationship between muscle glycogen availability at the end of recovery and time to exhaustion during Run-2. Incremental area under the concentration curve (iAUC) for plasma glucose and serum insulin concentrations during the recovery were calculated using the method recommended by Wolever (Wolever, 2004). Statistical procedures were performed using commercially available software (IBM SPSS version 21.0, SPSS Inc., Chicago, IL) and significance was set at an alpha level of 0.05. Unless otherwise stated, all results were reported in text as the mean  $\pm$  standard deviation (SD) of the mean and the error bars depicted in figures are confidence intervals (CI) that have been corrected to remove between-subject variance (Loftus and Masson, 1994a).

## Results

### Exercise capacity

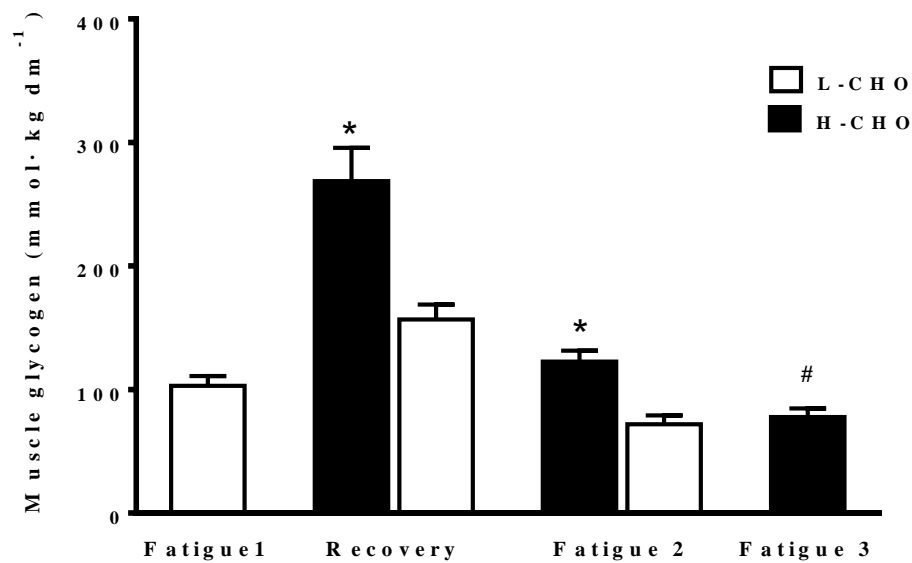
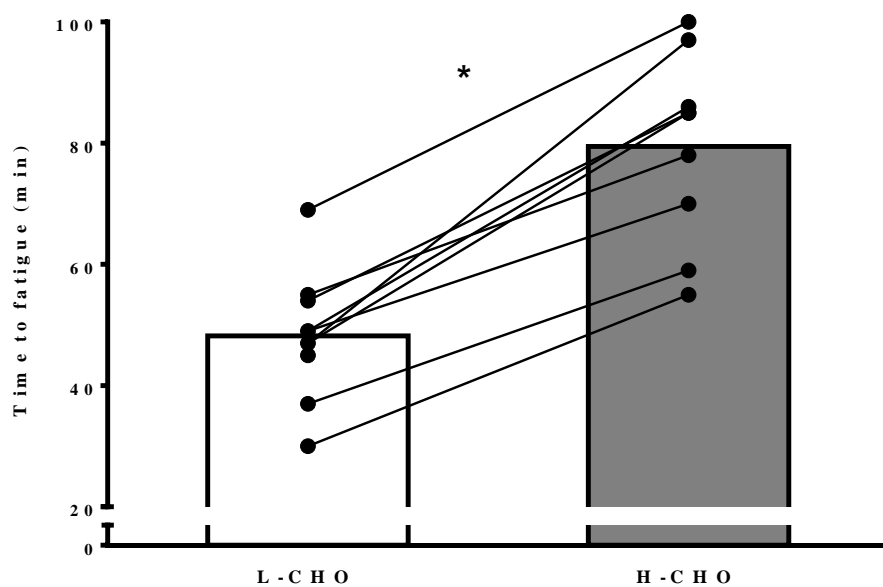
Run-1 and Run-2 times to exhaustion during the familiarisation trial were  $103 \pm 17$  min and  $36 \pm 9$  min, respectively. The run times to exhaustion in Run-1 (i.e. prior to intervention) were very well-matched between treatments, with median time to exhaustion of 105 min (72-133 min) in L-CHO trial and 105 min (75-161 min) in H-

CHO trial ( $p = 0.12$ ). All participants were able to run longer during the subsequent run when more carbohydrate had been ingested in recovery, with mean run times of  $48 \pm 11$  min in L-CHO and  $80 \pm 16$  min in H-CHO ( $p < 0.001$ ). Moreover, the magnitude of this pattern between treatments was consistent for every participant in the study (i.e. improvement of  $31 \pm 9$  min), as represented in Figure 4.2.

Relative exercise intensities were also successfully standardised between the experimental treatments and averaged  $69 \pm 1$  %  $\dot{V}O_{2\max}$  in Run-1 and  $69 \pm 3$  %  $\dot{V}O_{2\max}$  in Run-2 across both treatments. These were reflected by the overall heart rates of  $169 \pm 9$  and  $167 \pm 9$  beats·min<sup>-1</sup> recorded during L-CHO and H-CHO, respectively.

### Muscle glycogen

Figure 4.2 illustrates muscle glycogen concentrations across both treatments. A time  $\times$  trial interaction was established for total muscle glycogen concentrations ( $F = 9.8$ ;  $p = 0.003$ ) and accordingly there was greater muscle glycogen content at the end of recovery in H-CHO than L-CHO. Despite higher net glycogen degradation during Run-2 in the H-CHO treatment ( $200.1 \pm 86$  mmol·kg dm<sup>-1</sup>), when compared to the absolute fatigue time point in L-CHO trial ( $85.5 \pm 67$  mmol·kg dm<sup>-1</sup>;  $p = 0.05$ ) the muscle glycogen concentration at F2 was still higher in the former trial ( $123 \pm 28$  mmol·kg dm<sup>-1</sup> *versus*  $72 \pm 21$  mmol·kg dm<sup>-1</sup>;  $p < 0.01$ ). Muscle glycogen concentrations were reduced to similar levels at the point of volitional exhaustion in both trials (Figure 4.2). A significant correlation was established ( $r = 0.45$ ;  $p = 0.045$ ) between muscle glycogen content at the end of recovery and time to exhaustion during Run-2.

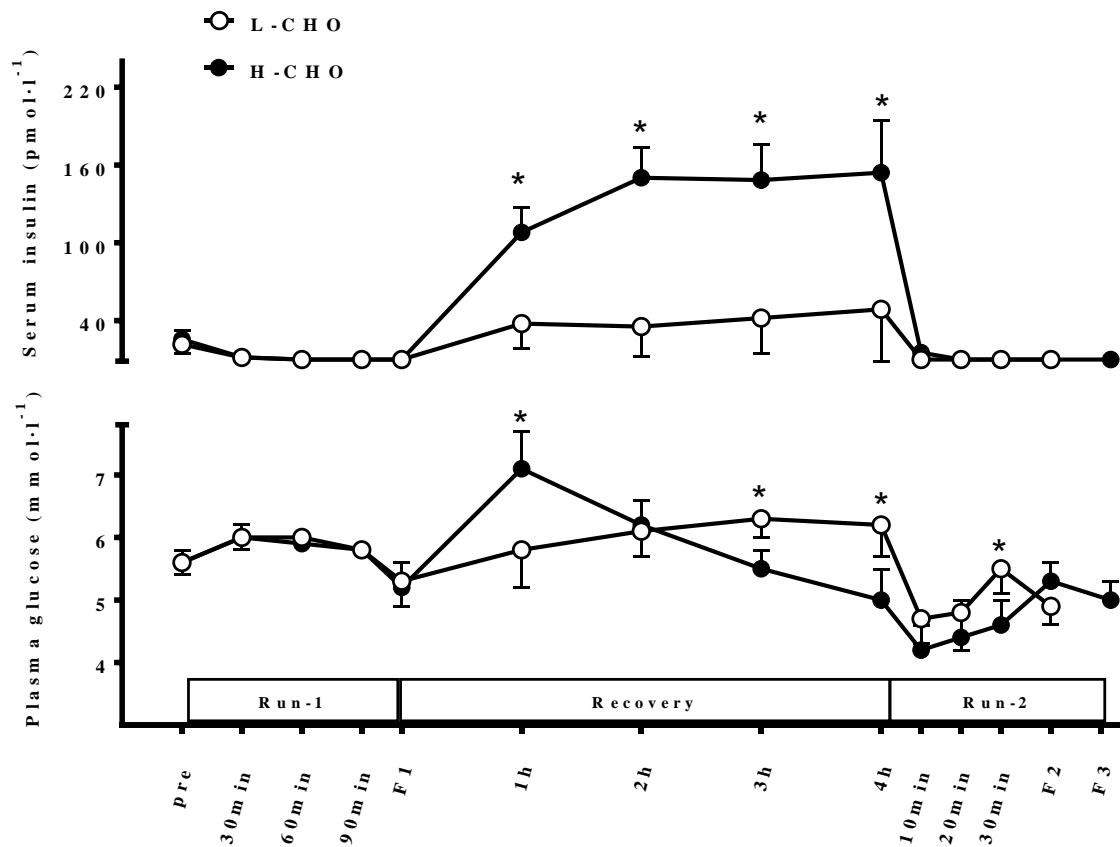
**A****B**

**Figure 4.2-A.** Muscle glycogen concentrations at the end of Run-1 (F1), at the end of 4 h recovery, time to exhaustion with L-CHO treatment (F2) and time to exhaustion with H-CHO treatment (F3). **Figure 4.2-B,** mean and individual run times to exhaustion following the ingestion of L-CHO or H-CHO during 4 h recovery. Values are means  $\pm$  CI. \*, values different between L-CHO and H-CHO ( $p < 0.01$ ). #, values different between F2 and F3 within the H-CHO treatment ( $p < 0.01$ ).

**Plasma glucose and serum insulin**

A time  $\times$  trial interaction was observed in plasma glucose during recovery ( $F= 8.65$ ;  $p= 0.004$ ; Figure 4.3), which was associated with a higher glycaemic iAUC in H-CHO ( $299 \pm 125 \text{ mmol}\cdot 240 \text{ min}\cdot\text{l}^{-1}$ ) during recovery than L-CHO ( $180 \pm 138 \text{ mmol}\cdot 240 \text{ min}\cdot\text{l}^{-1}$ ;  $p= 0.04$ ). There were also notable differences during the subsequent run ( $F= 5.63$ ;  $p= 0.02$ ), with slightly lower plasma glucose concentrations in H-CHO than L-CHO in the initial 30 min of exercise. No frank hypoglycaemia was observed at the point of fatigue in L-CHO ( $4.9 \pm 1.1 \text{ mmol}\cdot\text{l}^{-1}$ ) or H-CHO ( $5.0 \pm 0.9 \text{ mmol}\cdot\text{l}^{-1}$ ).

Serum insulin concentrations were significantly higher during recovery when H-CHO was ingested as opposed to L-CHO ( $F= 9.0$ ;  $p= 0.004$ ; Figure 4.3). Accordingly, the insulinemic iAUC for the entire 4-h recovery period was elevated threefold when H-CHO was ingested when compared to L-CHO ( $28 \pm 12$  *versus*  $7 \pm 3 \text{ nmol}\cdot 240 \text{ min}\cdot\text{l}^{-1}$ ;  $p= 0.02$ ).



**Figure 4.3.** Plasma glucose and serum insulin concentrations during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments. Values are mean  $\pm$  CI. \*, values different between L-CHO and H-CHO ( $p < 0.05$ ). F1, time to exhaustion during Run-1; F2, time to exhaustion with L-CHO treatment; F3= time to exhaustion with H-CHO treatment.

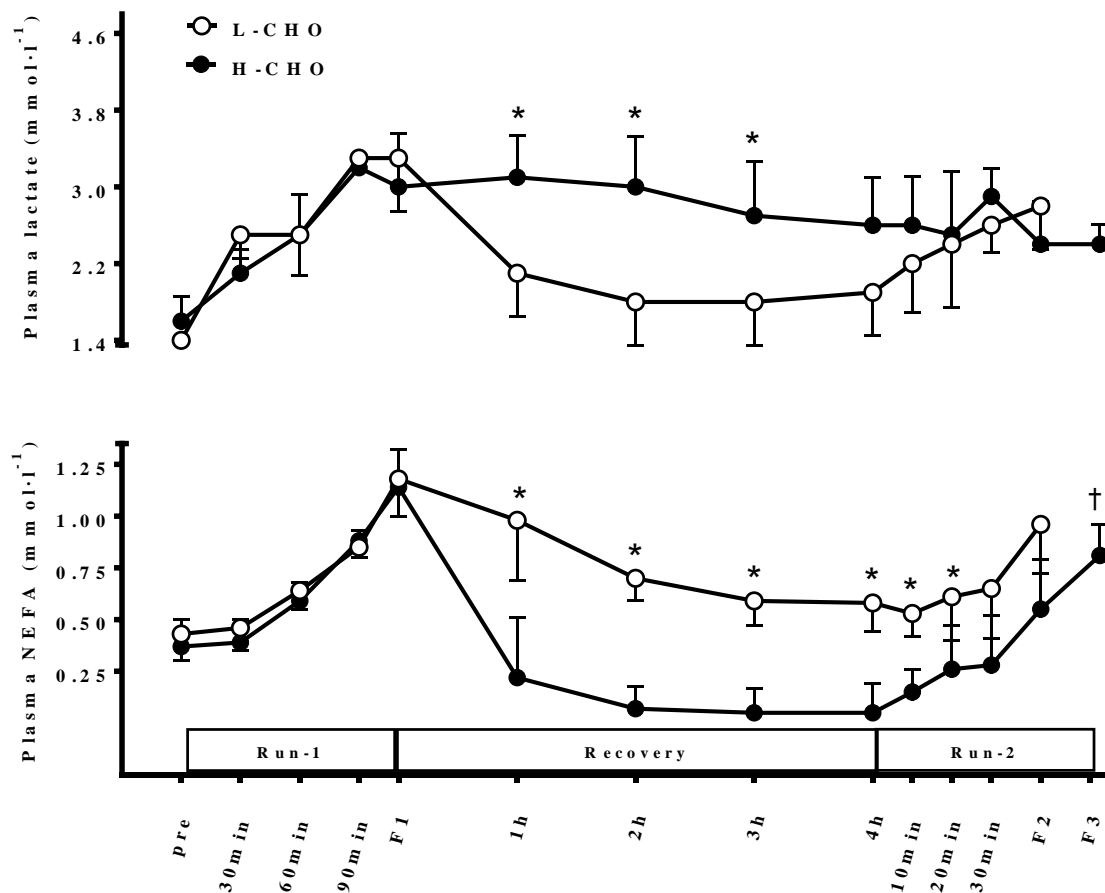
**Plasma NEFA and lactate**

Plasma NEFA concentrations were rapidly suppressed to basal levels during recovery in H-CHO while maintained at a relatively higher level in the L-CHO trial (treatment:  $p= 0.04$ ). Upon commencement of the subsequent run, plasma NEFA were consistently elevated in L-CHO when compared to H-CHO (treatment:  $p< 0.001$ ). An increase in NEFA concentrations from F2 to F3 was observed in H-CHO trial ( $p= 0.008$ , Figure 4.4).

Plasma lactate concentrations declined during recovery in L-CHO but remained relatively elevated in H-CHO (time:  $p= 0.005$ ). However, plasma lactate levels during the subsequent run (Figure 4.4) were not dissimilar between in L-CHO and H-CHO ( $2.5 \pm 0.3$  and  $2.6 \pm 0.2$  mmol·l<sup>-1</sup>, respectively;  $p= 0.6$ ).

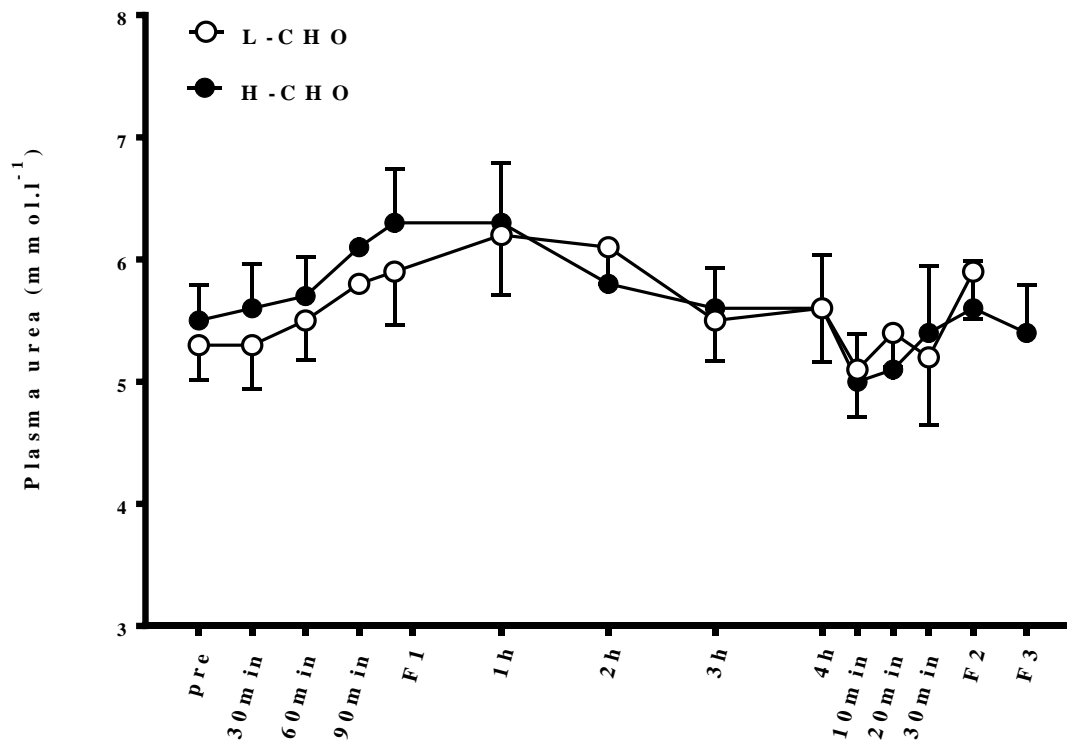
**Plasma urea**

The plasma concentration of urea was not different between treatments (Figure 4.5) and remained at basal levels throughout trials ( $5.6 \pm 0.4$  mmol·l<sup>-1</sup> in both treatments).



**Figure 4.4** Plasma NEFA and lactate concentrations during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments. Values are mean  $\pm$  CI. \*, values different between L-CHO and H-CHO ( $p < 0.01$ ). †, values different from F2 to F3 in H-CHO treatment. F1, time to exhaustion during Run-1; F2, time to exhaustion with L-CHO treatment; F3, time to exhaustion with H-CHO treatment.

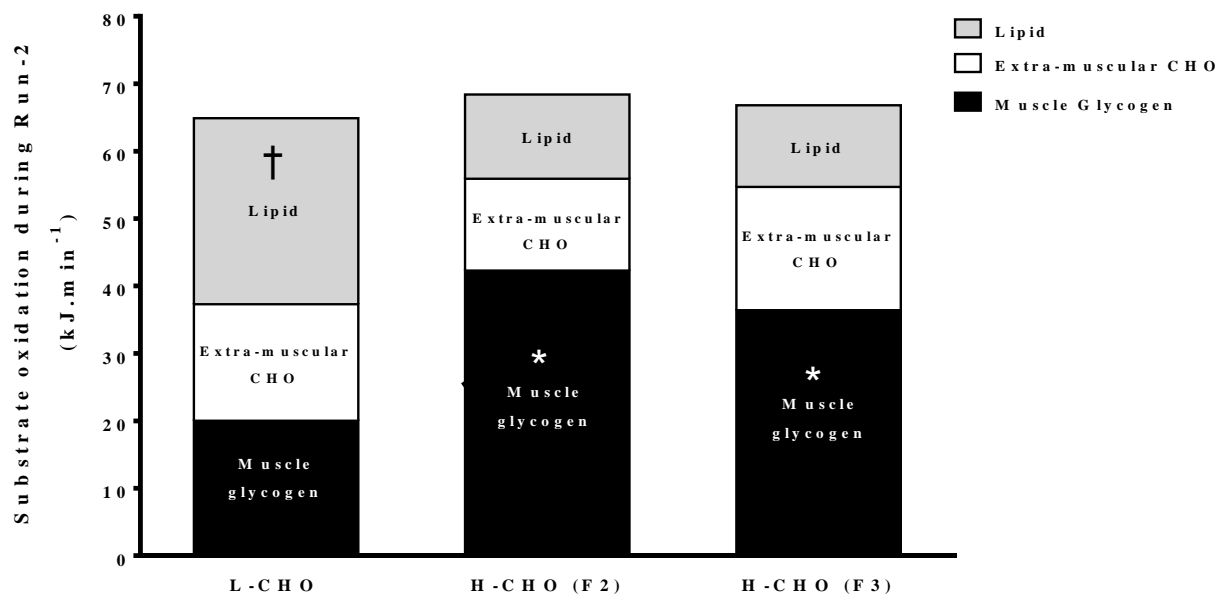




**Figure 4.5** Plasma urea during Run-1, recovery and Run-2 with L-CHO or H-CHO treatments. Values are mean  $\pm$  CI. F1, time to exhaustion during Run-1; F2, time to exhaustion with L-CHO treatment; F3, time to exhaustion with H-CHO treatment.

**Substrate metabolism**

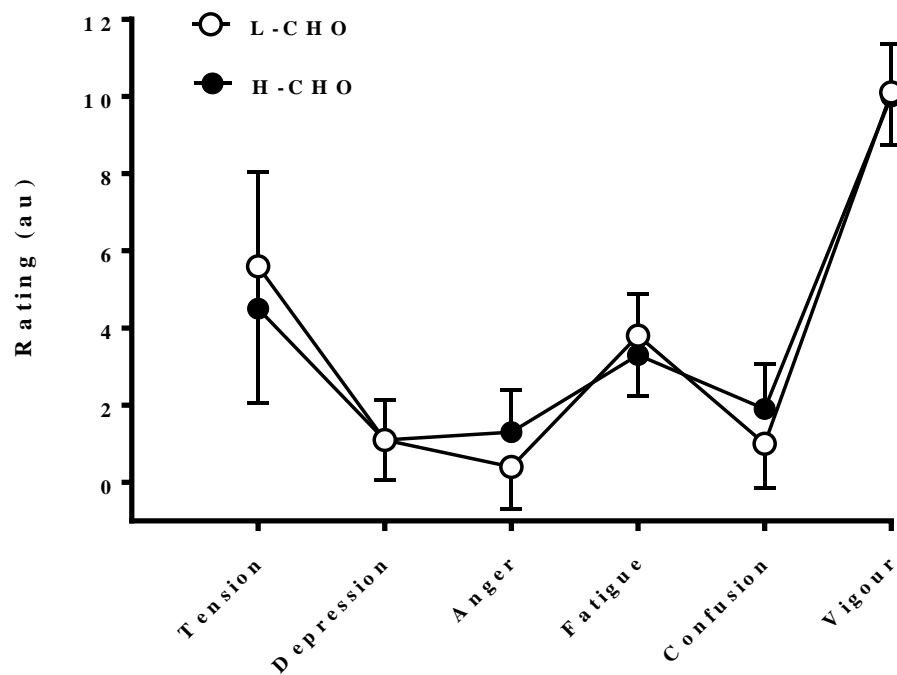
Whole-body carbohydrate and lipid oxidation rates were substantially different between treatments during Run-2 ( $F= 7.96$ ;  $p= 0.006$ ; Table 4.1). Although overall rates of metabolism during the repeated exercise bout were similar between treatments (L-CHO =  $64.9 \text{ kJ}\cdot\text{min}^{-1}$ ; H-CHO =  $66.7 \text{ kJ}\cdot\text{min}^{-1}$ ), H-CHO ingestion resulted in lower lipid oxidation rates than L-CHO ( $4.3 \pm 2.2$  vs.  $11.2 \pm 3.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ;  $p< 0.001$ ) but higher rates of carbohydrate oxidation ( $44.5 \pm 6.5$  vs.  $25.2 \pm 9.3 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ , respectively;  $p<0.001$ ). Figure 4.6 illustrates that the higher rates of whole body carbohydrate oxidation in H-CHO trial were likely attributable to variations in glycogen rather than extra-muscular carbohydrate metabolism (e.g. glucose and lactate), both at the point corresponding to fatigue with L-CHO (F2) and the point of absolute fatigue (F3).



**Figure 4.6** The contribution of muscle glycogen, extra-muscular carbohydrate (CHO) and lipids to total substrate metabolism ( $\text{kJ}\cdot\text{min}^{-1}$ ) during Run-2 with L-CHO or H-CHO treatments. \*, Muscle glycogen values different between L-CHO and H-CHO ( $p < 0.05$ ). †= lipid values different between L-CHO and H-CHO treatments. ( $p < 0.05$ ), F1, time to exhaustion during Run1; F2, time to exhaustion with L-CHO treatment; F3, time to exhaustion with H-CHO treatment.

**Hydration and subjective data**

Pre-exercise hydration status verified adequate fluid balance and was not different between treatments, as indicated by urine osmolality of  $496 \pm 316$  and  $540 \pm 266$  mOsm.kg<sup>-1</sup> in L-CHO and H-CHO, respectively ( $p= 0.5$ ). Changes in BM were similar ( $p= 0.6$ ) across both trials ( $-1.2 \pm 0.6$  and  $-1.3 \pm 0.6$  kg in L-CHO and H-CHO, respectively). The change in plasma volume was also similar ( $p= 0.9$ ) between the respective treatments ( $2 \pm 3$  vs.  $1.8 \pm 3$  %, respectively). The total urine produced during recovery was  $1749 \pm 840$  ml in L-CHO and  $1247 \pm 613$  ml in H-CHO trials ( $p= 0.09$ ). There were no differences in any of the mood state categories in the POMS-SF ( $p > 0.05$ ; Figure 4.7). A significant time x trial interaction was observed in RPE ( $F= 6.38$ ;  $p= 0.01$ ); participants' perceived effort was significantly lower in H-CHO than L-CHO from 15 min until F2 during Run-2 ( $p < 0.05$ ; Table 4.2). Subjective ratings of gut fullness, thirst, and stomach discomfort were similar between the experimental conditions (Table 4.3).



**Figure 4.7** Short form profile of mood state (POMS-SF) categories recorded before exercise with L-CHO and H-CHO treatments. Values are mean  $\pm$  CI. au, arbitrary units.

**Table 4.1** Substrate metabolism and respiratory exchange ratio (RER) during Run-1 and Run-2 with L-CHO or H-CHO treatments.

	Run-1					Run-2					
	Pre	30 min	60 min	90 min	F1	15 min	30 min	45 min	F2	60 min	F3
<b>Carbohydrate oxidation (g·min<sup>-1</sup>)</b>											
L-CHO	0.26±0.15	2.20±0.36	2.18±0.37	1.90 ±0.40	1.87±0.72	1.92±0.74	1.99±0.89	2.74±1.04	1.60±0.79		
						*	*		*		#
H-CHO	0.33±0.19	2.59±0.70	2.30±0.59	2.02±0.80	1.98±0.73	2.68±0.68	3.18±1.06	2.81±0.95	2.41±0.46	2.66±0.47	2.41±0.98
<b>Lipid oxidation (g·min<sup>-1</sup>)</b>											
L-CHO	0.06±0.06	0.57±0.20	0.61±0.21	0.76±0.22	0.77±0.36	0.77±0.22	0.71±0.31	0.56±0.31	0.94±0.24		
						*	*		*		#
H-CHO	0.06±0.07	0.44±0.20	0.57±0.23	0.66±0.44	0.70±0.32	0.24±0.17	0.26±0.18	0.40±0.26	0.45±0.16	0.36±0.23	0.50±0.33
<b>RER</b>											
L-CHO	0.87±0.12	0.89± 0.04	0.88±0.04	0.85±0.04	0.85±0.06	0.85±0.05	0.86±0.07	0.90±0.07	0.82±0.05		
						*	*		*		#
H-CHO	0.90±0.11	0.91±0.04	0.89±0.04	0.88±0.08	0.86±0.06	0.96±0.05	0.95±0.04	0.92±0.05	0.91±0.04	0.93±0.02	0.90±0.06

Values are mean ± SD. \*, values different between L-CHO and H-CHO (p< 0.05); #, values different at absolute fatigue (F2 vs. F3) between L-CHO and H-CHO (p<0.05).

**Table 4.2** Relative exercise intensities (%  $\dot{V}O_{2\max}$ ), ratings of perceived exertion (RPE) and heart rate during Run-1 and Run-2 in L-CHO and H-CHO treatments.

	Run-1					Run-2					
	Pre	30 min	60 min	90 min	F1	15 min	30 min	45 min	F2	60 min	F3
<b>% <math>\dot{V}O_{2\max}</math></b>											
L-CHO	—	68 ± 7	70 ± 5	71 ± 2	71 ± 5	72 ± 6	70 ± 7	70 ± 5	74 ± 5	—	—
H-CHO	—	68 ± 4	68 ± 5	69 ± 4	69 ± 4	69 ± 6	69 ± 6	67 ± 3	66 ± 5	67 ± 3	67 ± 5
<b>RPE (6-20)</b>											
L-CHO	—	13 ± 1	16 ± 2	18 ± 1	20 ± 0	15 ± 2	17 ± 2	19 ± 1	20 ± 0	—	—
H-CHO	—	12 ± 2	15 ± 2	17 ± 2	20 ± 0	13 ± 1*	15 ± 1*	16 ± 1*	16 ± 1*	18 ± 1	20 ± 0
<b>Heart rate (beats·min<sup>-1</sup>)</b>											
L-CHO	77 ± 12	174 ± 8	182 ± 9	184 ± 10	185 ± 8	175 ± 10	182 ± 11	181 ± 10	184 ± 8	—	—
H-CHO	77 ± 10	170 ± 7	176 ± 8	177 ± 9	180 ± 10	170 ± 11	177 ± 10	177 ± 8	175 ± 11	184 ± 3	179 ± 12

Values are mean ± SD.

**Table 4.3** Subjective measures of gut fullness, thirst and stomach discomfort following the ingestion of L-CHO and H-CHO.

	Recovery							
	0-h	0.5-h	1-h	1.5-h	2-h	2.5-h	3-h	3.5-h
<b>Gut fullness (scale 6-20)</b>								
L-CHO	8 ± 2	8 ± 2	9 ± 1	10 ± 3	10 ± 2	10 ± 3	10 ± 3	10 ± 3
H-CHO	7 ± 1	8 ± 2	9 ± 2	10 ± 3	10 ± 3	11 ± 4	10 ± 4	11 ± 4
<b>Thirst (scale 6-20)</b>								
L-CHO	11 ± 3	9 ± 2	9 ± 2	8 ± 2	8 ± 1	8 ± 1	7 ± 1	7 ± 1
H-CHO	11 ± 4	10 ± 3	9 ± 3	9 ± 2	8 ± 2	8 ± 2	7 ± 1	7 ± 1
<b>Stomach discomfort (scale 6-20)</b>								
L-CHO	8 ± 3	8 ± 2	8 ± 1	9 ± 2	9 ± 3	9 ± 3	9 ± 3	9 ± 3
H-CHO	8 ± 3	8 ± 1	8 ± 3	8 ± 3	9 ± 3	7 ± 1	9 ± 3	10 ± 4

Values are mean ± SD.



## Discussion

The experimental design presented here provides novel insight regarding the role of muscle glycogen in fatigue by enabling both time- and fatigue-matched comparisons of substrate availability and utilisation during the late stages of repeated exercise. Effective standardisation of other relevant variables lends direct support to the hypothesis that muscle glycogen availability after recovery from prior exercise is indeed a primary determinant of subsequent exercise capacity. From a practical perspective, having utilised nutritional manipulation of carbohydrate availability to understand the role of glycogen, it can also therefore be concluded that carbohydrate ingestion can be employed to impact repeated exercise capacity via this mechanism.

The improvement in subsequent endurance capacity with H-CHO treatment was clearly demonstrated by an increase of  $31 \pm 9$  min relative to L-CHO, which is in agreement with one previous experiment (Betts *et al.*, 2007) but in contrast with two others (Fallowfield and Williams, 1997; Wong and Williams, 2000). These discrepancies may be a consequence of a number of factors. The current study in addition to that of Betts *et al.* (Betts *et al.*, 2007) included younger participants with higher  $\dot{V}O_{2\max}$  than those used in previous investigations (Fallowfield and Williams, 1997; Wong and Williams, 2000). Furthermore, we employed a familiarisation trial that was identical to the main experimental procedures. These measures may be an important distinction when considering that aerobically trained individuals who are familiarised with exercise capacity testing may be necessary to detect small, worthwhile intervention effects (Hopkins *et al.*, 2001). Moreover, subtle differences in the current experimental procedures may have contributed to accurately reaching true volitional exhaustion. Specifically, participants in the current experiment, as well as the only other study reporting a dose-dependent improvement in exercise capacity with carbohydrate ingestion (Betts *et al.*, 2007), reduced the intensity on two occasions before fatigue was accepted. Indeed, participants were able to run for  $10 \pm 4$  min from the first walk until the point of exhaustion in this study, enforcing the notion that volitional exhaustion may not have been achieved in previous investigations that did not allow these walks. Of course, other differences between protocols such as the precise type, amount, and/or feeding frequency of the ingested carbohydrate offer

possible alternative explanations (Fallowfield and Williams, 1997; Wong and Williams, 2000).

Ingestion of  $1.2 \text{ g sucrose} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  markedly increased muscle glycogen availability compared to the relatively low quantity of sucrose ( $0.3 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). This finding is consistent with most previous studies investigating muscle glycogen restoration with differing amounts of carbohydrate (Betts and Williams, 2010). In the current experiment, muscle glycogen utilisation was accelerated with higher carbohydrate intake and thus glycogenolysis was shown to be proportional to muscle glycogen concentration, as has previously been determined (Arkinstall *et al.*, 2004; Shearer *et al.*, 2001). Nevertheless, similar rates of muscle glycogen utilisation were reported during a repeated exercise bout when differing amounts of carbohydrate were ingested during recovery (Berardi *et al.*, 2006; Tsintzas *et al.*, 2003). The precise reasons for these apparently discrepant findings in relation to muscle glycogen utilisation may be ascribed to the use of  $^{13}\text{C}$ -magnetic resonance spectroscopy by Berardi *et al.* (2006) to quantify muscle glycogen degradation (i.e. wider musculature *versus* biochemical analysis of  $<100 \text{ mg}$  from the *vastus lateralis*; although these techniques correlate well (Taylor *et al.*, 1992)) and the type of exercise performed (i.e. cycling) that were dissimilar from the present study. Equally, the study by Tsintzas *et al.* (2003) employed treadmill running during a non-exhaustive exercise bout (15 min) and provided lower amounts of carbohydrate to the current experiment ( $0.15 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  *versus*  $0.53 \text{ g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). Concurrent with our finding that muscle glycogen concentrations were reduced to similar levels at the point of volitional exhaustion across both treatments, the current data suggest that muscle glycogen availability *per se* was associated with the improved restoration of endurance capacity with increased carbohydrate intake.

When interpreting the ergogenic effect with H-CHO ingestion, it is important to consider the brief period where exercise was interrupted in this trial to obtain a muscle biopsy sample to compare glycogen utilisation at this fatigue-matching point relative to L-CHO. Muscle glycogen restoration would occur at very low rates in the absence of carbohydrate feeding ( $\approx 0.5 \text{ mmol} \cdot \text{kg dry mass}^{-1} \cdot \text{min}^{-1}$  (Ivy *et al.*, 1988a)). During a

subsequent exercise bout at similar intensities, muscle glycogen utilisation was estimated to be  $\approx 2.5 \text{ mmol} \cdot \text{kg dry mass}^{-1} \cdot \text{min}^{-1}$  during treadmill running (Tsintzas *et al.*, 2003). Thus, any resynthesis that may have occurred during the brief interruption period ( $624 \pm 236$  seconds) would theoretically account for only 2 min of extended exercise. Other possibilities that may have influenced subsequent exercise capacity in H-CHO treatment include knowledge of the treatment order and the psychological impact of resting period to obtain the muscle biopsy. Nevertheless, regarding the former, it was previously demonstrated that there was no placebo effect when carbohydrate was ingested during prolonged cycling, and that there was a clear ergogenic effect with carbohydrate intake relative to both a placebo and water ingestion (Hulston and Jeukendrup, 2009). In relation to the psychological effect of the brief period to obtain the muscle sample, it was apparent that participants were able to continue exercising during H-CHO (RPE;  $16 \pm 1$ ) relative to the fatigue-matching point (i.e. F2) in L-CHO ( $20 \pm 0$ ) and thus indicating that participants' perceived effort was lower in H-CHO than L-CHO treatment before exercise was interrupted in H-CHO to obtain the final biopsy sample. When considered collectively, it is reasonable to affirm that the short period to obtain a muscle sample is unlikely to explain the 65 % improvement in the capacity for subsequent exercise and that the imposed nutritional intervention may be ascribed for the ergogenic effect with H-CHO intake.

The lowering of blood glucose was more prominent in H-CHO during the initial 30 min of the subsequent run, likely reflecting a transient increase in leg glucose uptake and reduced liver glucose output secondary to the increase in insulin concentrations (Marmy-Conus *et al.*, 1996). Conversely, the relatively elevated plasma glucose concentrations early in exercise in the L-CHO trial likely reflect an increased hepatic glucose output, which is predominantly supported by an increased rate of hepatic glycogenolysis (Wahren *et al.*, 1971). Thus, the increased insulinemic response during recovery in H-CHO may have initially spared liver glycogenolysis such that glucose production to the active muscles was possible late in exercise. These physiological responses coupled with our finding of limited muscle glycogen restoration in L-CHO supports our prior assumption that the modest amounts of ingested carbohydrate will be largely sequestered by the liver due to highly efficient first pass hepatic extraction

(Casey *et al.*, 2000; Wasserman *et al.*, 1991). It is likely that liver glycogen resynthesis was augmented in both trials owing to the presence of fructose in the sucrose solutions (Delarue *et al.*, 1993) and thus the ongoing absorption of the ingested carbohydrate in H-CHO treatment is likely to contribute to the observed higher carbohydrate oxidation with this treatment. Indeed, both liver and muscle glycogen have an important role in restoration of subsequent endurance capacity (Casey *et al.*, 2000). Therefore, it is not unreasonable to suggest that liver glycogen availability and increased exogenous carbohydrate oxidation may have contributed to the overall effect in the H-CHO treatment. Nonetheless, estimations of extra-muscular carbohydrate oxidation were not different between F2 and F3 in the H-CHO treatment (Figure 4.6). In conjunction with the observation of an increased net glycogen utilisation with H-CHO and that fatigue in both treatments coincided with depletion of muscle glycogen to critically low muscle glycogen concentrations, the current findings demonstrate that the availability of muscle glycogen is a primary determinant of fatigue during a repeated exercise bout following short-term recovery.

Hypoglycaemia and subsequent reduction in carbohydrate oxidation late in exercise have been proposed as a major cause of fatigue during prolonged moderate to high-intensity cycling exercise (Coyle *et al.*, 1986). However, it has been consistently demonstrated that fatigue during prolonged moderate to high-intensity running is not associated with hypoglycaemia (Tsintzas and Williams, 1998; Tsintzas *et al.*, 1996a; Tsintzas *et al.*, 2001). The latter notion was further supported by the current investigation, whereby fatigue was not associated with hypoglycaemia in either treatment. Additionally, whilst carbohydrate oxidation during a repeated exercise bout was greater when higher amounts of carbohydrate ( $\approx 0.75 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) were provided relative to a lower dose ( $\approx 0.25 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ ) during recovery, no discernible differences in plasma glucose concentrations or time to exhaustion were shown (Fallowfield and Williams, 1997). Indeed, fatigue during prolonged exercise was shown independent of carbohydrate oxidation or avoidance of hypoglycaemia (Claassen *et al.*, 2005). Further support for the latter study comes from H-CHO trial in the present investigation, where neither hypoglycaemia nor a decline in carbohydrate oxidation was apparent at the cessation of exercise to explain fatigue. Thus, it can be suggested

that factors other than hypoglycaemia or a decline in carbohydrate oxidation rates limited the capacity for subsequent exercise.

In conclusion, the ingestion of  $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  of carbohydrate during 4 h recovery from an initial exhaustive exercise bout increased muscle glycogen availability prior to a repeated exercise bout when compared with the ingestion of  $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ . In concordance, the capacity for repeated exercise was improved in a dose-dependent manner. Net muscle glycogen utilisation was accelerated in the H-CHO trial during the repeated exercise bout and fatigue was associated with glycogen depletion to critically low levels in both treatments. The extended run time to fatigue expected with increasing carbohydrate intake is attributable to increased muscle glycogen repletion during recovery and therefore the availability of this substrate during Run-2.

## CHAPTER 5

### INFLUENCE OF POST-EXERCISE PROTEIN INGESTION ON MUSCLE GLYCOGEN METABOLISM IN RECOVERY AND SUBSEQUENT RUNNING EXERCISE

#### Introduction

Muscle glycogen is an important substrate supporting the energetic requirements of prolonged moderate to high-intensity exercise (van Loon *et al.*, 2001). Given the well-established close relationship between the perception of fatigue and muscle glycogen depletion to critically low levels, the capacity to perform physical exercise is determined to a large extent by the availability of glycogen at the onset of exercise (Bergstrom *et al.*, 1967). In concordance, recovery following exercise is mainly determined by the time required to replenish endogenous carbohydrate reserves and adequate carbohydrate ingestion is required to maximise this process (Jentjens and Jeukendrup, 2003). However, when the time available for recovery from glycogen-depleting exercise is limited ( $\leq 8$  h), neither glycogen replenishment nor the capacity for subsequent exercise are likely to be fully restored, highlighting the requirement for nutritional strategies to accelerate recovery in preparation for repeated exercise (Betts and Williams, 2010). While it is known that carbohydrate ingestion at a rate of  $\approx 1.2$  g·kg BM<sup>-1</sup>·h<sup>-1</sup> maximises muscle glycogen repletion during short-term recovery, other aspects of post-exercise nutrition such as tissue reconditioning and adaptation are also important for optimal recovery (Betts and Williams, 2010). The latter would require the ingestion of protein to stimulate muscle protein synthesis and mediate tissue repair/reconditioning, and thus it is now generally recommended that post-exercise nutrition for endurance-type exercise should include both carbohydrate and protein (Beelen *et al.*, 2010).

The addition of protein to post-exercise carbohydrate has been suggested to increase the efficiency of muscle glycogen storage from a prior exercise bout relative to when only carbohydrate is ingested (Berardi *et al.*, 2006; Ivy *et al.*, 2002; Zawadzki *et al.*,

1992). However, considerable disparity exists regarding the potential for protein co-ingestion to facilitate muscle glycogen restoration, potentially due to variance in the mode of exercise performed, degree of prior muscle glycogen depletion, and/or the dose/type of carbohydrate/protein ingested during recovery (Betts and Williams, 2010). Indeed, some experiments have demonstrated a glycogenic effect of adding protein to a carbohydrate solution even when the energy content is matched between treatments (Berardi *et al.*, 2006; Ivy *et al.*, 2002), yet others do not verify such benefits (Beelen *et al.*, 2012; Ferguson-Stegall *et al.*, 2011b; Howarth *et al.*, 2009; Lunn *et al.*, 2012). Although the addition of protein to carbohydrate is unlikely to further increase muscle glycogen repletion above that of an energy-matched carbohydrate supplement (Ferguson-Stegall *et al.*, 2011b; van Loon *et al.*, 2000b), it remains possible that co-ingesting protein with carbohydrate during short-term recovery can maximise muscle glycogen resynthesis when insufficient amounts of carbohydrate (i.e.  $\leq 1 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) are ingested (Detko *et al.*, 2013; van Loon *et al.*, 2000b). Nevertheless, whether the co-ingestion of protein with carbohydrate can elicit maximal muscle glycogen resynthesis rates relative to an energy-matched carbohydrate provided in amounts ( $\geq 1 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) sufficient to maximise short-term recovery from running has not been determined. In fact, only two studies have examined glycogen restoration following a standardised bout of running-based exercise (Betts *et al.*, 2008; Lunn *et al.*, 2012), and neither explored these effects after an initial exhaustive exercise bout. By definition, prolonged exercise to exhaustion elicits a greater magnitude of muscle glycogen depletion, which in turn regulates insulin sensitivity, glucose uptake, glycogen synthase activity and ultimately muscle glycogen storage (Jentjens and Jeukendrup, 2003; Zachwieja *et al.*, 1991).

Beyond the above potential for carbohydrate and protein co-ingestion to impact glycogen resynthesis in post-exercise recovery, both carbohydrate alone and when co-ingested with protein have the potential to independently improve the capacity for subsequent exercise (Betts *et al.*, 2007; Lunn *et al.*, 2012; Thomas *et al.*, 2009; Williams *et al.*, 2003), although these findings are not universal (Betts *et al.*, 2005). The mechanism behind the ergogenic effect of added protein apparent in some studies therefore remains undefined, but a reduced rate of glycogen use during repeated exercise has been implicated/proposed (Betts *et al.*, 2007; Betts *et al.*, 2008; Ivy *et al.*,

2003). Although pre-exercise whey protein hydrolysate ingestion was recently shown to attenuate glycogen degradation during exhaustive exercise bout in rodents (Morifuji *et al.*, 2011), no study has examined the effects of added protein during recovery from previous exercise on muscle metabolism during subsequent exercise in humans. Therefore, the previous reports that carbohydrate-protein ingestion improves subsequent exercise capacity/performance relative to energy-matched carbohydrate supplement (Ferguson-Stegall *et al.*, 2011b; Lunn *et al.*, 2012; Thomas *et al.*, 2009) may be explained through this physiological mechanism.

To this end, the current study explored the effects of ingesting carbohydrate with added protein on muscle glycogen metabolism following exhaustive running and during a repeated exercise test to exhaustion. It was hypothesised that: i) carbohydrate-protein ingestion would be as effective as an isocaloric carbohydrate supplement to maximise muscle glycogen repletion; ii) carbohydrate-protein ingestion would not alter muscle glycogen degradation and thus subsequent endurance capacity would not be enhanced; and iii) fatigue in both treatments would be a consequence of muscle glycogen depletion to critically low levels.

## Materials and Methods

### Participants

Six recreationally active runners (5 men and 1 woman) participated in the study. The characteristics of the cohort were: age  $26 \pm 11$  years; body mass (BM)  $66.5 \pm 7.9$  kg; height  $180 \pm 5$  cm;  $\dot{V}O_{2\max}$   $64 \pm 4$  ml·kg<sup>-1</sup>·min<sup>-1</sup>; weekly exercise duration  $5 \pm 3$  h. The participants were informed about the possible risks and discomforts involved before giving their voluntary consent to take part. The study was approved by the local National Health Services Research Ethics Committee (Ref: 09/H0101/82) with a controlled clinical trial number: ISRCTN87937960.



### Experimental design

Each participant was required to perform two main trials applied in double-blinded, counterbalanced randomized experimental design interspersed by an interval of  $\geq 2$  weeks. A weighed dietary record was completed 48 h preceding the familiarisation trial, and was subsequently replicated before to the main trials ( $11405 \pm 1305 \text{ kJ}\cdot\text{d}^{-1}$ ;  $49 \pm 2 \%$  carbohydrate;  $35 \pm 2 \%$  fat;  $16 \pm 2 \%$  protein). Participants were provided with a standardised meal ( $3180 \text{ kJ}$ ;  $57 \%$  carbohydrate;  $24 \%$  Protein;  $19 \%$  fat) in the evening ( $12 \pm 1 \text{ h}$ ) before the familiarisation trial and replicated this prior to each main trial. Participants were also requested to abstain from alcohol consumption and refrain from strenuous physical activity for 48 h pre-trial, with any light exercise recorded and matched during the period of standardisation of lifestyle for ensuing trials.

The main trials required participants to run to the point of volitional fatigue (Run-1) at an intensity of  $70 \%$   $\dot{V}\text{O}_{2\text{max}}$  followed by a 4 h recovery period, where participants ingested a carbohydrate-protein (CHO-P) or an isocaloric carbohydrate (CHO) supplement. Following recovery, a second run time to fatigue (Run-2) with a similar exercise intensity to the first run (i.e.  $70 \%$   $\dot{V}\text{O}_{2\text{max}}$ ) was undertaken to assess participants' exercise capacity following each nutritional provision (Figure 5.1). Muscle biopsies were obtained at the start and end of recovery to assess the rate of muscle glycogen resynthesis over this period. A third muscle biopsy was taken at the point of volitional exhaustion during Run-2 to examine the net muscle glycogen degradation during the repeated exercise bout.

### Experimental protocol

Each participant arrived to the laboratory at  $08:00 \pm 1 \text{ h}$  following an overnight fast ( $\geq 10 \text{ h}$ ). Upon arrival to the laboratory, participants completed a profile of mood state (POMS) questionnaire, before a mid-flow urine sample was obtained to assess hydration. Post-void nude BM was then recorded (Weylux, UK) before 5 min resting expired gas sample was collected. An indwelling cannula was inserted into an antecubital vein and a 10 ml baseline venous blood sample was obtained. The cannula

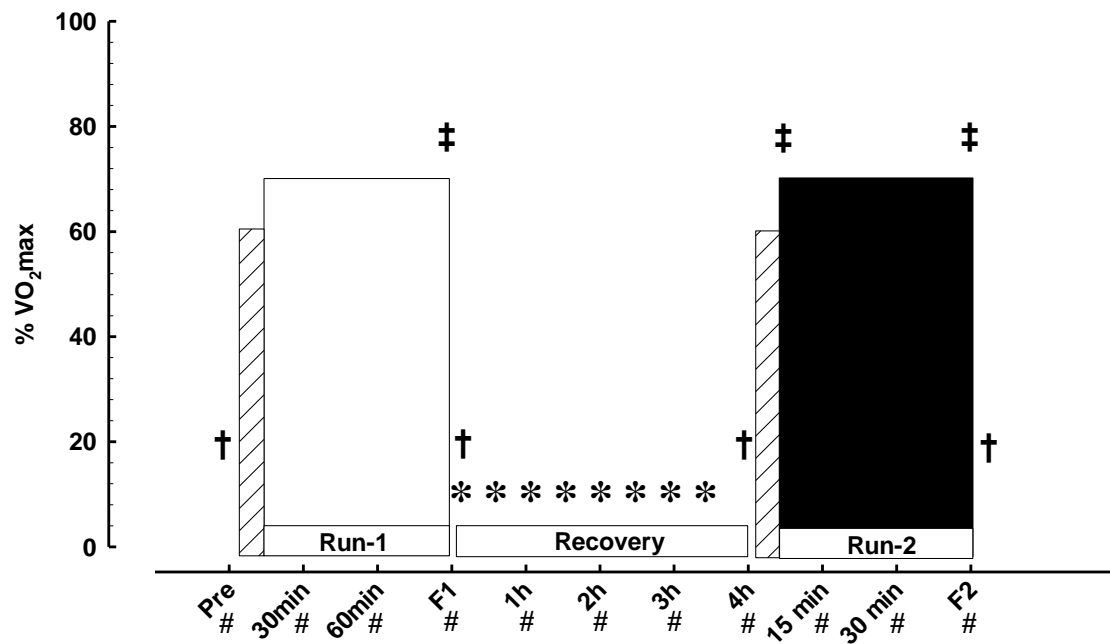
was kept patent by frequent flushing with isotonic saline immediately after any blood collection throughout the trial. A 3-5 mm incision was made at the anterior aspect of the thigh using a surgical blade under local anaesthetic (1 % lidocaine; Hameln Pharmaceuticals Ltd., Brockworth, UK) and the biopsy site was dressed to obtain a muscle sample following Run-1. Prior to mounting the treadmill, resting heart rate (HR) was recorded (Polar FT2, Kempele, Finland), and participants commenced the exercise protocol with a standardised 5 min warm-up at 60 %  $\dot{V}O_{2\max}$ , where speed was then increased to 70 %  $\dot{V}O_{2\max}$  until the point of volitional exhaustion. During Run-1, one minute expired gas samples, HR, ratings of perceived exertion (RPE) and 10 ml venous blood samples were collected (Figure 5.1). Water intake was permitted *ad libitum* during the familiarisation trial ( $0.4 \pm 0.3$  L during Run-1) and then matched for subsequent trials. To accurately gauge relative levels of fatigue, participants were permitted to reduce the intensity (walking at  $4.4 \text{ km} \cdot \text{h}^{-1}$ ) for 2 min intervals when they indicated that they could not maintain the prescribed intensity. Only on the third occasion when participants indicated that they were unable to run at the prescribed speed was volitional exhaustion accepted, and a final minute expired gas and venous blood samples were collected and time to fatigue recorded (minus the time elapsed during the walking intervals). Immediately following Run-1, participants were instructed to rest on an adjacent bed in a semi-supine position and a  $\approx 80$  mg muscle biopsy sample was obtained from the *vastus lateralis* using percutaneous needle biopsy technique (Bergstrom, 1962), with the order of dominant/non-dominant legs for muscle biopsy sampling being counterbalanced between the main trials. Thereafter, the first bolus of prescribed solution was immediately provided (see section below) and recovery time commenced, before nude BM was recorded to assess hydration status through percentage change in mass (with BM adjusted for the ingested bolus).

The remaining seven aliquots of prescribed solution were provided in 30 min intervals (Figure 5.1). Subjective measures of stomach discomfort, gut fullness and thirst were recorded following the ingestion of each bolus using adapted Borg scales (Borg, 1973). Expired gas followed by venous blood samples were collected hourly prior to fluid provision. Furthermore, urine output from each participant was collected throughout the 4 h recovery period. Approximately 3 h 40 min from the first biopsy

sample, two further 3-5 mm incisions were made proximally relative to the first incision site, which were taken from the same leg at least 3 cm apart. Nude BM was recorded at the end of recovery, and participants initiated a standardised warm-up before running at 70 %  $\dot{V}O_{2\max}$  to the point of volitional exhaustion. As for Run-1, water intake was permitted *ad libitum* during the familiarisation trial and matched for subsequent trials ( $0.3 \pm 0.3$  L during Run-2). The criteria to achieve volitional exhaustion were identical to the initial exercise bout. Expired gases, venous blood, HR and RPE were collected at different intervals (Figure 5.1). Immediately following Run-2, the remaining incision site was used to obtain a final muscle biopsy during both treatments. BM was subsequently recorded following the attainment of the final biopsy from each participant. Ambient temperature and humidity were recorded at 60 min intervals throughout the trials using a portable weather station (WS 6730; Technoline, Berlin, Germany) and were not different among the trials:  $20.3 \pm 0.5$  and  $20.1 \pm 0.5$  °C; and  $46 \pm 2$  and  $47 \pm 2$  % in L-CHO and H-CHO trials, respectively. Background music was standardised between trials and participants were unaware of the time elapsed during the exercise capacity test, with all verbal encouragement standardised.

### **Solution composition**

The source of carbohydrate was sucrose in both CHO and CHO-P treatments. The solutions were provided in equal volumes and relative to each participant's BM (mean  $\pm$  SD;  $665 \pm 80$  ml·h<sup>-1</sup>) and the rate of carbohydrate intake in CHO was 1.2 g·kg BM<sup>-1</sup>·h<sup>-1</sup>, providing a total amount of carbohydrate of  $319 \pm 38$  g. In the CHO-P trial, 0.8 g·kg BM<sup>-1</sup>·h<sup>-1</sup> of carbohydrate was provided with an additional 0.4 g·kg BM<sup>-1</sup>·h<sup>-1</sup> of whey protein hydrolysate, which equates to a total of  $213 \pm 25$  g of carbohydrate and  $106 \pm 13$  g of protein during the entire recovery period. Both supplements were matched for their electrolyte content (sodium and potassium) and flavour (calorie-free vanilla extract; Flavdrops, Myprotein, UK). Pretesting was conducted to ensure that these supplements were matched for flavour. Blinding was verified using a formal exit interview, based upon which only two participants were able to distinguish between treatments.



**Figure 5.1** A schematic representation of the study protocol. †, body mass assessment; \*, fluid provision; #, expired gas and blood sample; ‡, muscle biopsy; F1, fatigue in Run-1; F2, fatigue in Run-2; dashed columns, warm-up; clear column, run time to exhaustion in Run-1; black column, run time to exhaustion in Run-2.

### Statistical analysis

*A priori* sample size estimation was conducted based on the exercise capacity data of a similar previous study (Betts *et al.*, 2007) which showed that a sample size of  $n=6$  produced 85 % power to detect a difference in exercise capacity of 7.5 min using a two-tailed *t*-test between carbohydrate and a carbohydrate plus protein supplements. Paired *t*-tests were used to analyse data involving a single comparison of two level means. Where data deemed non-normally distributed, values are reported as median (range), with Wilcoxon signed rank test being used to compare medians. A two-way linear mixed model with repeated measures (time  $\times$  trial) was employed to identify overall differences between experimental conditions with trial and time inserted as covariates. Where significant interaction effect was found a Bonferroni step-wise correction was employed to determine the location of the variance (Atkinson, 2002a). Incremental area under the concentration curve (iAUC) for plasma glucose and serum insulin concentrations during the recovery period were calculated using the method recommended by Wolever (Wolever, 2004). Statistical procedures were performed using commercially available software (IBM SPSS version 21.0, SPSS Inc., Chicago, IL) and significance was set at an alpha level of 0.05. Unless otherwise stated, all results are reported as the mean  $\pm$  standard deviation (SD) of the mean and the error bars depicted in figures are confidence intervals (CI) that have been corrected to remove between-subject variance (Loftus and Masson, 1994a).

## Results

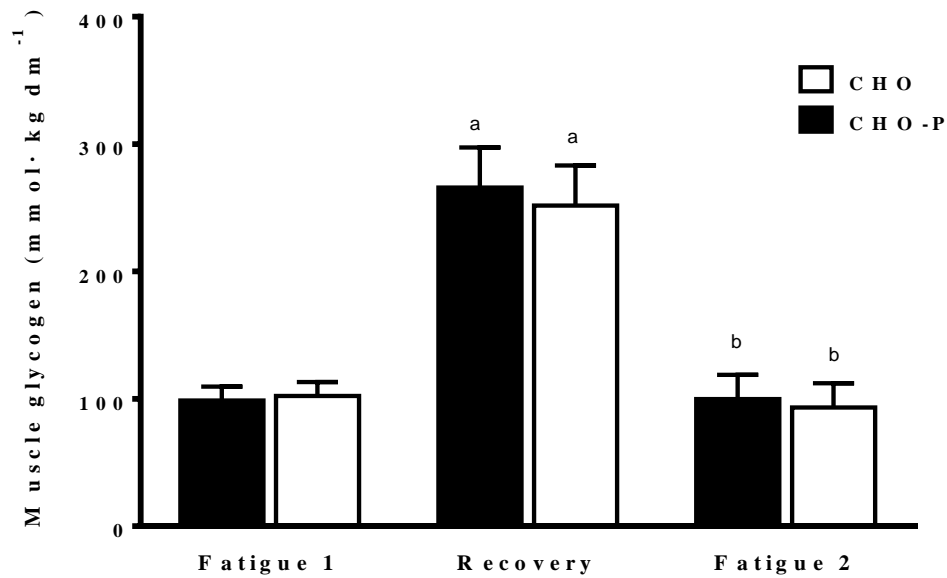
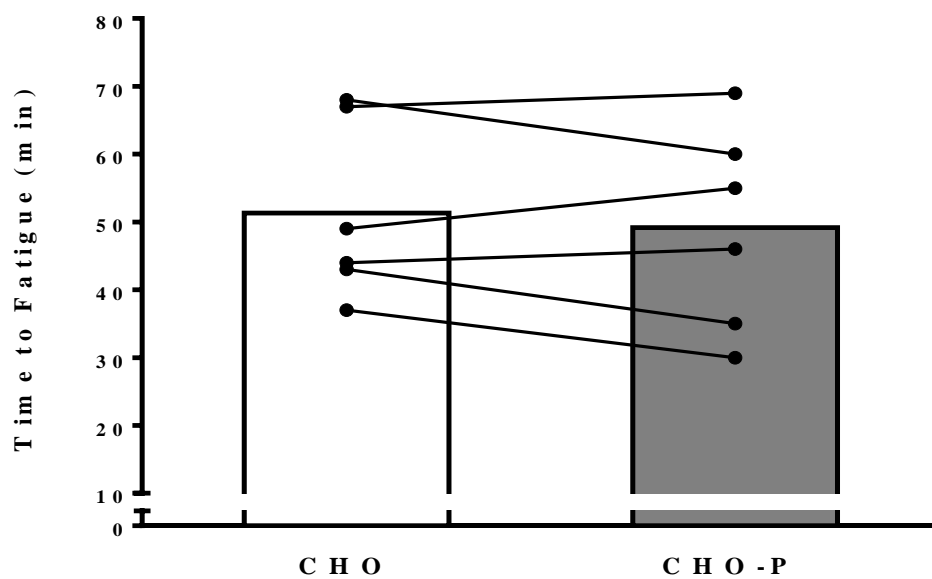
### Exercise capacity

The run times to exhaustion in Run-1 (i.e. prior to intervention) were well-matched between treatments, with mean time to exhaustion of  $81 \pm 17$  min in CHO and  $84 \pm 19$  min in CHO-P ( $p=0.37$ ). No differences were observed during the subsequent run, with mean run times of  $51 \pm 13$  min in CHO and  $49 \pm 15$  min in CHO-P ( $p=0.43$ ). The individual running times are presented in Figure 5.2.

Relative exercise intensities were also successfully standardised between the experimental treatments and averaged  $69 \pm 1 \%$   $\dot{V}O_{2\max}$  in Run-1 and  $70 \pm 1 \%$   $\dot{V}O_{2\max}$  in Run-2 across both treatments. These were reflected by the overall similar heart rates of  $173 \pm 3$  and  $172 \pm 4$  beats $\cdot$ min $^{-1}$  recorded during CHO and CHO-P, respectively.

### **Muscle glycogen**

Figure 5.2 illustrates muscle glycogen concentrations across both treatments. No time  $\times$  trial interaction was identified for total muscle glycogen concentrations ( $F= 0.08$ ;  $p= 0.79$ ). The concentration of muscle glycogen was not different between treatments following Run-1 ( $99 \pm 3$  mmol $\cdot$ kg dm $^{-1}$ ). A higher muscle glycogen content at the end of recovery was demonstrated in both treatments in response to feeding (time:  $p< 0.001$ ). Muscle glycogen repletion in the 4 h recovery period was  $149.5 \pm 59$  mmol $\cdot$ kg dm $^{-1}$  in CHO and  $166.8 \pm 27$  mmol $\cdot$ kg dm $^{-1}$  in CHO-P ( $p= 0.47$ ). Net muscle glycogen degradation during Run-2 was similar between trials ( $158.6 \pm 33$  *versus*  $163.1 \pm 29$  mmol $\cdot$ kg dm $^{-1}$  in CHO and CHO-P, respectively;  $p= 0.31$ ). Muscle glycogen concentrations were reduced to similar levels ( $97 \pm 5$  mmol $\cdot$ kg dm $^{-1}$ ) at the point of volitional exhaustion during the subsequent run in both trials (Figure 5.2).

**A****B**

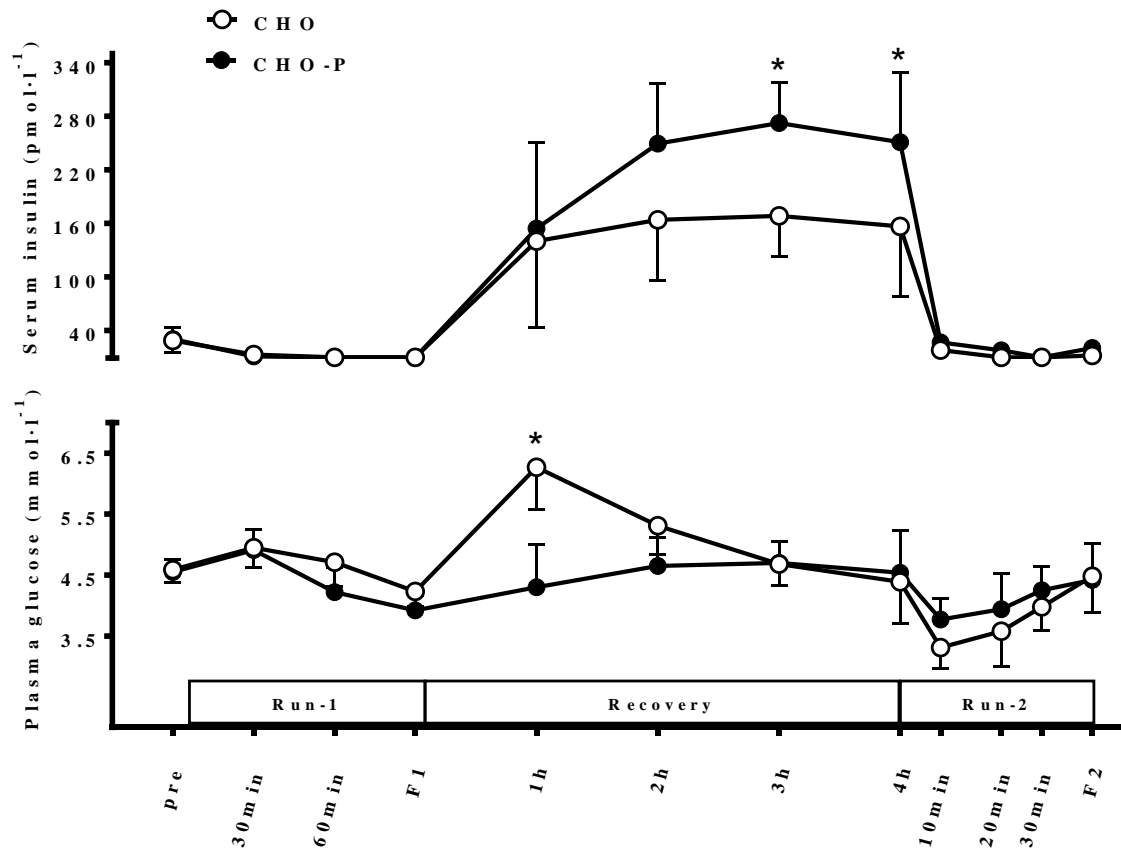
**Figure 5.2-A** Muscle glycogen concentrations at the end of Run-1 (Fatigue 1), at the end of 4 h recovery, and the end of Run-2 (Fatigue 2) with CHO and CHO-P treatments. Values are means  $\pm$  CI. **Figure 5.2-B**, mean and individual run times to exhaustion during Run 2 following the ingestion of CHO or CHO-P during 4 h recovery. a, values different from fatigue at Run-1 to post-recovery ( $p < 0.01$ ); b, values different from post-recovery to fatigue at Run-2 ( $p < 0.01$ ).

**Plasma glucose and serum insulin**

A time x trial interaction was observed in plasma glucose during recovery ( $F= 3.9$ ;  $p= 0.05$ ; Figure 5.3). During recovery, plasma glucose concentrations were higher in CHO than CHO-P at 1 h, with no notable difference in any other time point during this period (Figure 5.3). Conversion of these data into a glycaemic iAUC for the entire 4 h recovery, however, did not reveal any differences ( $230 \pm 89$  *versus*  $186 \pm 163$   $\text{mmol} \cdot 240 \text{ min} \cdot \text{l}^{-1}$ ;  $p= 0.42$ ) between CHO and CHO-P, respectively. Similarly, there were no notable differences in plasma glucose concentrations between treatments during Run-2 ( $F= 0.22$ ;  $p= 0.64$ ). No frank hypoglycaemia was observed at the point of fatigue in CHO ( $4.5 \pm 0.8 \text{ mmol} \cdot \text{l}^{-1}$ ) or CHO-P ( $4.4 \pm 0.3 \text{ mmol} \cdot \text{l}^{-1}$ ).

Serum insulin concentrations were significantly higher during recovery when CHO-P was ingested as opposed to CHO ( $F= 3.6$ ;  $p= 0.05$ ; Figure 5.3). Accordingly, the insulinemic iAUC for the entire 4-h recovery period was greater when CHO-P was ingested when compared to CHO ( $47 \pm 18$  *versus*  $31 \pm 7$   $\text{nmol} \cdot 240 \text{ min} \cdot \text{l}^{-1}$ ;  $p= 0.05$ ). However, there were no differences in serum insulin concentrations between treatments during Run-2.





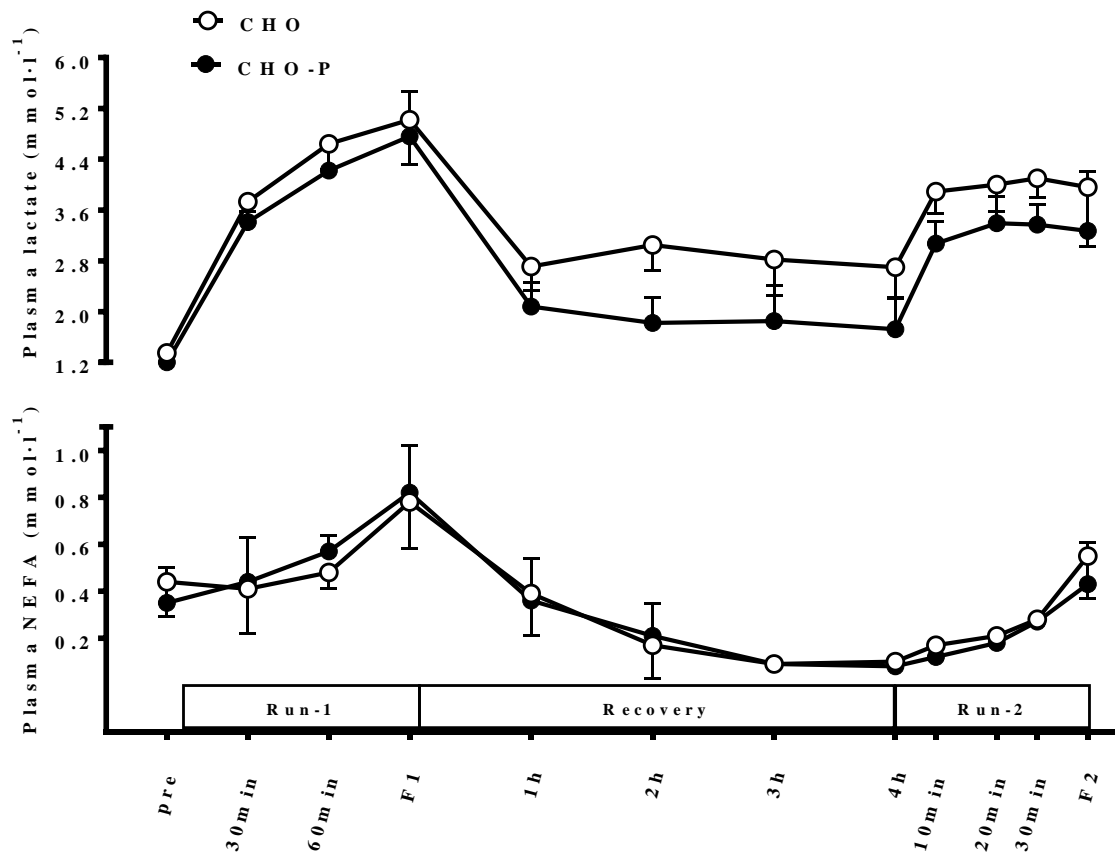
**Figure 5.3** Plasma glucose and serum insulin concentrations during Run-1, recovery and Run-2 with CHO or CHO-P treatments. Values are mean  $\pm$  SE. \*, values different between CHO and CHO-P ( $p < 0.05$ ). F1, time to exhaustion during Run-1; F2, time to exhaustion during Run-2.

**Plasma NEFA and lactate**

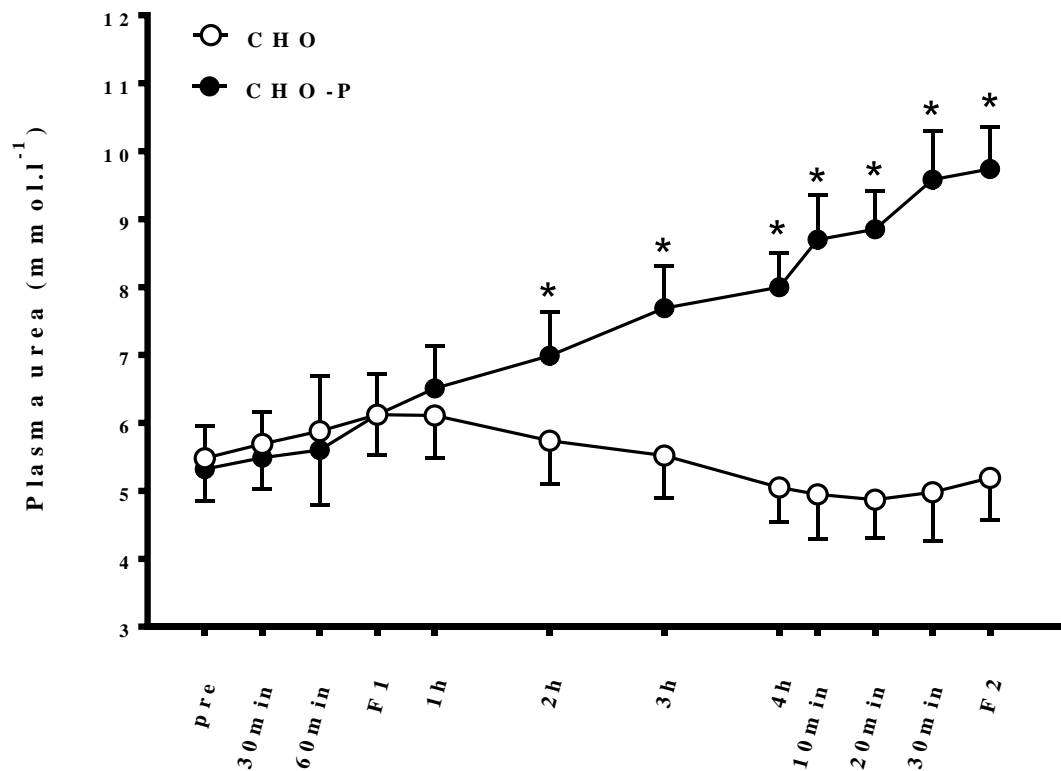
Plasma lactate concentrations declined during recovery in CHO-P but remained relatively elevated in CHO. While plasma lactate levels during the subsequent run appeared to be slightly elevated in CHO than CHO-P ( $3.9 \pm 0.9$  and  $3.3 \pm 0.2$ , respectively) no time x trial interactions were identified ( $F= 78$ ;  $p= 0.38$ ; Figure 5.4).

**Plasma urea**

There was a marked increase in plasma urea concentration following the ingestion of protein in the CHO-P trial, while plasma urea remained at basal level in CHO ( $F: 91.8$ ;  $p < 0.001$ ). This gradual increase persisted until the end of the trial, reaching  $9.7 \pm 3.7$  mmol·l<sup>-1</sup> at the point of fatigue with CHO-P ingestion relative to  $5.2 \pm 1.0$  mmol·l<sup>-1</sup> in CHO treatment ( $p < 0.001$ ; Figure 5.5).



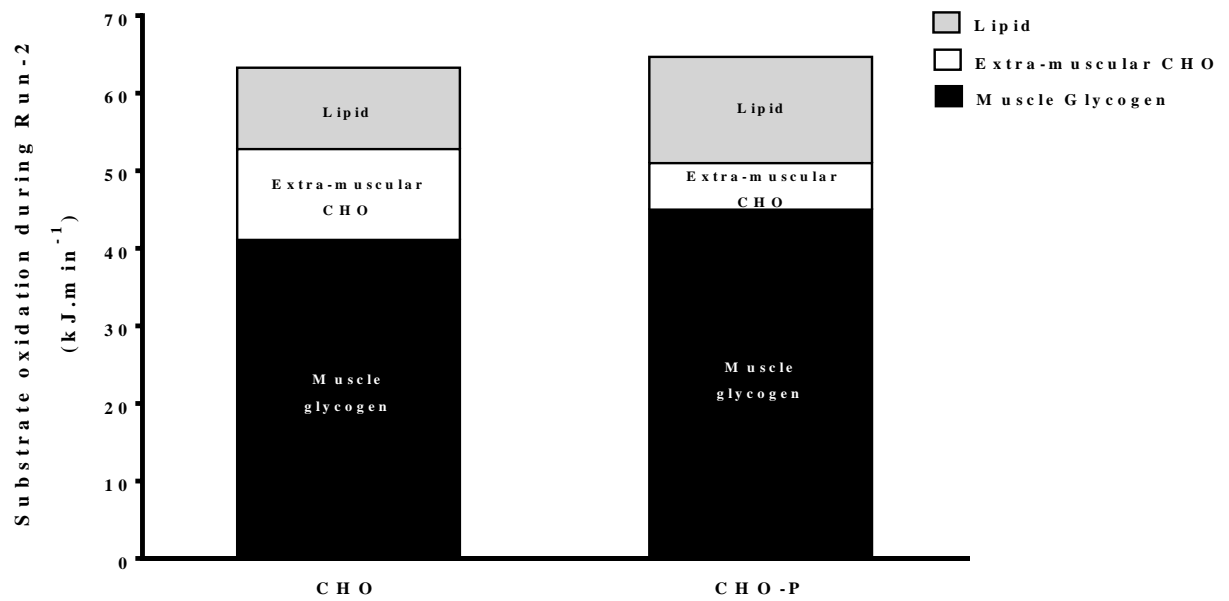
**Figure 5.4** Plasma NEFA and lactate concentrations during Run-1, recovery and Run-2 with CHO or CHO-P treatments. Values are mean  $\pm$  CI. \*, values different between CHO and CHO-P ( $p < 0.05$ ). F1, time to exhaustion during Run-1; F2, time to exhaustion during Run-2.



**Figure 5.5** Plasma urea during Run-1, recovery and Run-2 with CHO or CHO-P treatments. Values are mean  $\pm$  CI. \*, values different between CHO and CHO-P ( $p < 0.05$ ). F1, time to exhaustion during Run-1; F2, time to exhaustion during Run-2.

**Substrate metabolism**

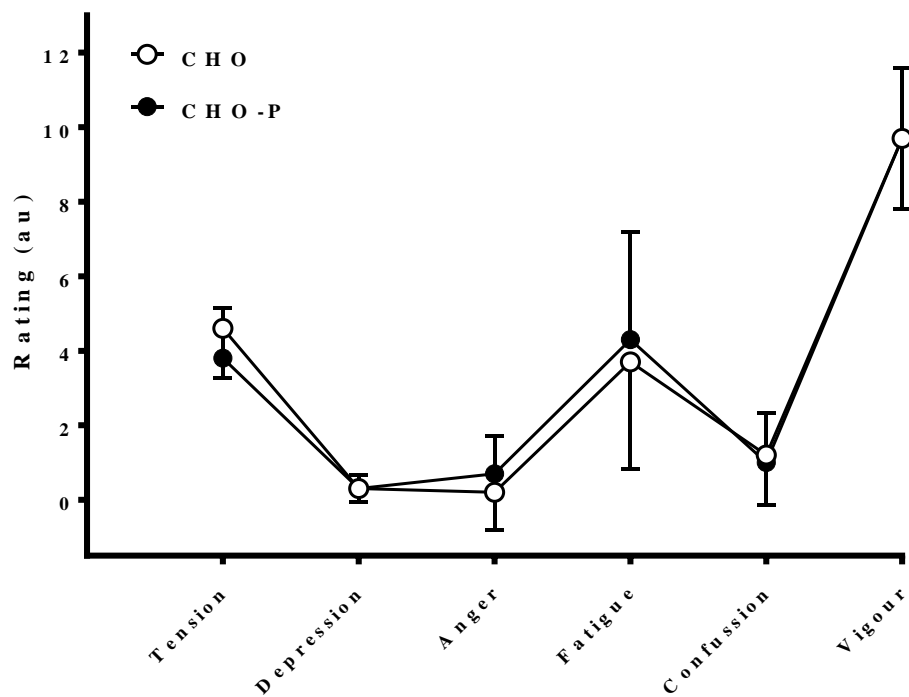
Whole-body carbohydrate ( $F=0.002$ ;  $p=0.97$ ) and lipid ( $F=0.012$ ;  $p=0.91$ ) oxidation rates were not different between treatments during recovery (Table 5.1). Rates of metabolism during the repeated exercise bout were similar between treatments (CHO=  $63.2 \text{ kJ}\cdot\text{min}^{-1}$ ; CHO-P=  $64.5 \text{ kJ}\cdot\text{min}^{-1}$ ). Neither lipid oxidation ( $4.1 \pm 2.4$  *versus*  $5.3 \pm 3.9 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) nor carbohydrate oxidation ( $44.0 \pm 9.2$  *versus*  $41.4 \pm 9.3 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) rates were different during subsequent exercise between CHO and CHO-P, respectively ( $p=0.20$ ). Figure 5.6 illustrates that the contribution of muscle glycogen, extra-muscular carbohydrate (e.g. glucose and lactate) and lipids to substrate metabolism during Run-2 and indicates the comparable substrate selection between treatments during the repeated exhaustive exercise bout.



**Figure 5.6** The contribution of muscle glycogen, extra-muscular carbohydrate (CHO) and lipids to total substrate metabolism ( $\text{kJ}\cdot\text{min}^{-1}$ ) during Run-2 with CHO or CHO-P.

**Hydration and subjective data**

Pre-exercise hydration status verified adequate fluid balance and was not different between treatments, as indicated by urine osmolality of  $594 \pm 280$  and  $483 \pm 275$  mOsm.kg<sup>-1</sup> in CHO and CHO-P, respectively ( $p = 0.5$ ). Changes in BM were similar ( $p = 0.6$ ) across both trials ( $-1.0 \pm 0.7$  and  $-1.1 \pm 0.8$  kg in CHO and CHO-P, respectively). The change in plasma volume was also similar ( $p = 0.8$ ) between the respective treatments ( $0.1 \pm 5$  versus  $0.1 \pm 5$  %, respectively). The total urine produced during recovery was  $783 \pm 302$  ml in CHO and  $600 \pm 393$  ml in CHO-P trials ( $p = 0.2$ ). There were no differences in any of the mood state categories in the POMS-SF ( $p > 0.05$ ; Figure 5.7). Overall RPE scores were consistent between trials ( $17 \pm 2$  and  $17 \pm 2$ ; Table 5.2). Subjective ratings of gut fullness, thirst, and stomach discomfort and were similar between the experimental conditions (Table 5.3).



**Figure 5.7** Short form profile of mood state (POMS-SF) categories recorded before exercise with L-CHO and H-CHO treatments. Values are mean  $\pm$  SE. au, arbitrary units.



**Table 5.1** Substrate metabolism and respiratory exchange ratio (RER) during Run-1, recovery and Run-2 with CHO or CHO-P treatments.

	Run-1				Recovery				Run-2		
	Pre	30 min	60 min	F1	1 h	2h	3 h	4 h	15 min	30 min	F2
<b>Carbohydrate oxidation (<math>\text{g}\cdot\text{min}^{-1}</math>)</b>											
CHO	0.15 $\pm$ 0.07	1.97 $\pm$ 0.05	2.02 $\pm$ 0.58	2.35 $\pm$ 0.85	0.29 $\pm$ 0.18	0.33 $\pm$ 0.19	0.30 $\pm$ 0.15	0.33 $\pm$ 0.20	2.74 $\pm$ 0.52	2.88 $\pm$ 0.41	3.07 $\pm$ 1.10
CHO-P	0.19 $\pm$ 0.13	2.05 $\pm$ 0.40	2.20 $\pm$ 0.75	2.37 $\pm$ 0.89	0.23 $\pm$ 0.19	0.20 $\pm$ 0.11	0.33 $\pm$ 0.19	0.29 $\pm$ 0.13	2.71 $\pm$ 0.55	2.31 $\pm$ 0.24	2.82 $\pm$ 0.86
<b>Lipid oxidation (<math>\text{g}\cdot\text{min}^{-1}</math>)</b>											
CHO	0.05 $\pm$ 0.05	0.46 $\pm$ 0.22	0.58 $\pm$ 0.20	0.63 $\pm$ 0.30	0.07 $\pm$ 0.03	0.03 $\pm$ 0.02	0.03 $\pm$ 0.02	0.05 $\pm$ 0.03	0.33 $\pm$ 0.19	0.26 $\pm$ 0.15	0.20 $\pm$ 0.29
CHO-P	0.06 $\pm$ 0.05	0.52 $\pm$ 0.27	0.55 $\pm$ 0.31	0.67 $\pm$ 0.43	0.10 $\pm$ 0.03	0.11 $\pm$ 0.02	0.09 $\pm$ 0.03	0.09 $\pm$ 0.03	0.37 $\pm$ 0.20	0.51 $\pm$ 0.30	0.34 $\pm$ 0.38
<b>RER</b>											
	<u><b>Non-protein respiratory exchange ratios</b></u>										
CHO	0.80 $\pm$ 0.06	0.87 $\pm$ 0.04	0.87 $\pm$ 0.05	0.91 $\pm$ 0.08	0.89 $\pm$ 0.14	1.04 $\pm$ 0.40	1.00 $\pm$ 0.26	0.99 $\pm$ 0.19	0.93 $\pm$ 0.04	0.95 $\pm$ 0.03	0.96 $\pm$ 0.08
CHO-P	0.80 $\pm$ 0.06	0.88 $\pm$ 0.03	0.89 $\pm$ 0.06	0.92 $\pm$ 0.05	0.84 $\pm$ 0.21	0.81 $\pm$ 0.14	0.84 $\pm$ 0.12	0.89 $\pm$ 0.27	0.93 $\pm$ 0.03	0.90 $\pm$ 0.04	0.95 $\pm$ 0.09

Values are mean  $\pm$  SD.

**Table 5.2** Relative exercise intensities (%  $\dot{V}O_{2\max}$ ), ratings of perceived exertion (RPE) and heart rate during Run-1 and Run-2 in CHO and CHO-P treatments.

	Run-1				Run-2		
	Pre	30 min	60 min	F1	15 min	30 min	F2
<b>% <math>\dot{V}O_{2\max}</math></b>							
H-CHO	—	67 ± 3	68 ± 1	69 ± 2	68 ± 3	70 ± 4	71 ± 4
CHO-P	—	67 ± 2	69 ± 2	69 ± 2	69 ± 3	70 ± 4	72 ± 4
<b>RPE (6-20)</b>							
H-CHO	—	14 ± 1	18 ± 2	20 ± 0	15 ± 2	17 ± 2	20 ± 0
CHO-P	—	14 ± 1	17 ± 3	20 ± 0	15 ± 2	17 ± 2	20 ± 0
<b>Heart rate (beats·min<sup>-1</sup>)</b>							
H-CHO	63 ± 10	169 ± 9	176 ± 9	176 ± 7	170 ± 8	173 ± 9	173 ± 9
CHO-P	67 ± 2	166 ± 5	175 ± 7	175 ± 6	169 ± 4	173 ± 6	174 ± 5

Values are mean ± SD.

**Table 5.3** Subjective measures of gut fullness, thirst and stomach discomfort following the ingestion of CHO and CHO-P.

	Recovery							
	0-h	0.5-h	1-h	1.5-h	2-h	2.5-h	3-h	3.5-h
<b>Gut fullness (scale 6-20)</b>								
H-CHO	9 ± 3	10 ± 3	10 ± 3	10 ± 3	11 ± 3	11 ± 3	12 ± 3	13 ± 3
CHO-P	8 ± 2	9 ± 2	10 ± 2	10 ± 2	10 ± 2	11 ± 2	11 ± 2	11 ± 2
<b>Thirst (scale 6-20)</b>								
H-CHO	12 ± 3	12 ± 3	11 ± 2	10 ± 2	10 ± 2	9 ± 1	9 ± 2	8 ± 1
CHO-P	12 ± 4	11 ± 3	11 ± 3	10 ± 2	10 ± 3	10 ± 3	9 ± 3	9 ± 3
<b>Stomach discomfort (scale 6-20)</b>								
H-CHO	8 ± 2	8 ± 2	8 ± 2	8 ± 1	10 ± 3	10 ± 3	11 ± 3	12 ± 3
CHO-P	8 ± 1	8 ± 1	8 ± 1	8 ± 1	9 ± 1	9 ± 1	9 ± 2	10 ± 2

**Values are mean ± SD.**

## Discussion

This experiment demonstrates that co-ingesting  $0.4 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  of whey protein hydrolysate with  $0.8 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  carbohydrate during short-term recovery from exhaustive running exercise promotes muscle glycogen resynthesis at the same high rate as an isocaloric solution providing carbohydrate-only at the recommended ingestion rate (i.e.  $1.2 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$ ). Moreover, the inclusion of protein did not alter the metabolic response during the second bout nor therefore further improve subsequent exercise capacity. This is the first study to report data pertaining to muscle glycogen resynthesis following an exhaustive running exercise bout and also the first to report net glycogen degradation during repeated exercise following ingestion of carbohydrate and protein during recovery.

Co-ingesting protein with carbohydrate has been reported to enhance subsequent exercise capacity (Betts *et al.*, 2007; Lunn *et al.*, 2012; Thomas *et al.*, 2009; Williams *et al.*, 2003) and performance (Ferguson-Stegall *et al.*, 2011b), although others show no such effect (Betts *et al.*, 2005). Importantly, no plausible mechanism for the ergogenic benefit of carbohydrate-protein ingestion has been confirmed, albeit a number of potential candidates have been proposed in relation to an initial exercise bout. For example, the inclusion of protein may provide precursors for *de novo* replenishment of tricarboxylic acid cycle (TCA) intermediates and therefore flux in the skeletal muscle, although a depleted TCA intermediate pool in the working muscle does not compromise aerobic provision (Gibala *et al.*, 2002). Another suggested mechanism is that protein co-ingestion may impact central fatigue. Whilst there is some evidence that amino acid availability can reduce perceptions of fatigue during exercise (Blomstrand *et al.*, 1997), it remains debatable whether the inclusion of protein with carbohydrate can improve exercise performance through this mechanism (van Hall *et al.*, 1995). Another potential mechanism that has been suggested is that the addition of protein may result in reduced muscle glycogen use during exercise after recovery (Ivy *et al.*, 2003); although it was subsequently confirmed that added protein does not alter muscle glycogen degradation in humans when ingested alongside carbohydrate during an initial prolonged exercise bout

(Cermak *et al.*, 2009). However, all the potential mechanisms detailed above relate to an initial exercise bout, whereas the mechanisms of fatigue during a repeated exercise bout within hours of the first remain largely unexplored. In fact, only one investigation examined muscle glycogen metabolism during repeated exhaustive exercise bouts and demonstrates that the capacity for repeated exercise is mainly determined by the availability of muscle glycogen at the end of recovery (Alghannam *et al.*, 2015). Thus, the current investigation is the first to directly explore muscle glycogen degradation during repeated exercise when protein was added in the preceding recovery and directly shows that carbohydrate-protein ingestion does not alter muscle glycogen degradation during this bout relative to an isocaloric carbohydrate solution, consistent with the absence of any ergogenic effect.

When considered collectively, the inconsistencies regarding the ergogenic effect of protein co-ingestion on a repeated exercise bout may be associated with differences in total energy intake during recovery irrespective of macronutrient composition. In support of this contention, an ergogenic effect was shown when  $0.3 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  of protein was added to a  $0.8 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  carbohydrate solution; however, the restoration of endurance capacity was similar between an isocaloric carbohydrate ( $1.1 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$ ) and the carbohydrate-protein mixture (Betts *et al.*, 2007). In line with this reasoning, the restoration of endurance capacity may inherently be dependent on muscle glycogen availability prior to the repeated exercise bout. It has been established that pre-exercise muscle glycogen availability determines the capacity for exercise during an initial bout such that fatigue often corresponds with critically low ( $\approx 100 \text{ mmol}\cdot\text{kg dm}^{-1}$ ) muscle glycogen levels (Bergstrom *et al.*, 1967). This was evident in the current experiment, whereby substrate metabolism and time to fatigue during Run-2 was well-matched between trials, and the point of fatigue in both treatments was associated with low muscle glycogen concentrations (Figure 5.2). Nonetheless, dissociation between muscle glycogen resynthesis rates during recovery and subsequent exercise have been reported, such that improvements in exercise capacity/performance were shown with protein co-ingestion despite similar glycogen resynthesis rates during recovery (Ferguson-Stegall *et al.*, 2011b; Lunn *et al.*, 2012). However, these seemingly equivocal findings may be attributable to the provision of chocolate milk, which includes other nutrients such as caffeine that may mediate

independent effects on exercise performance regardless of the protein fraction (Hulston and Jeukendrup, 2008). Furthermore, the study by Lunn *et al.* (2012) employed a capacity test that induced fatigue within  $\approx 3$  min (Lunn *et al.*, 2012), suggesting that factors other than glycogen-dependent mechanisms were responsible for the termination of exercise (Betts *et al.*, 2005). It can therefore be concluded that the results of the current study are in agreement with our previous report (Alghannam *et al.*, 2015) in that muscle glycogen availability at the end of short-term recovery is the major determinant of subsequent exercise capacity.

Given the potent role of insulin in stimulating post-exercise muscle glucose uptake and muscle glycogen resynthesis (Jentjens and Jeukendrup, 2003), it is noteworthy that a higher insulin concentration was shown with protein co-ingestion despite that muscle glycogen resynthesis rates were not different between treatments. A higher insulin response to carbohydrate-protein ingestion with no concurrent increase in muscle glycogen has been reported previously (Beelen *et al.*, 2012; Betts *et al.*, 2008; van Hall *et al.*, 2000). Conversely, greater muscle glycogen restoration was synchronous with higher insulin levels in other reports (Williams *et al.*, 2003; Zawadzki *et al.*, 1992). These apparently discrepant findings are likely to be related to the precise amount of carbohydrate provided during recovery. As such, studies showing a concomitant increase in muscle glycogen storage with greater insulin levels typically provided carbohydrate in amounts lower than that suggested to maximise muscle glycogen resynthesis (i.e.  $< 1$  g carbohydrate $\cdot$ kg BM $^{-1}\cdot$ h $^{-1}$ ) during short-term recovery (Williams *et al.*, 2003; Zawadzki *et al.*, 1992). In contrast, when carbohydrate was ingested at higher intakes ( $> 1$  g carbohydrate $\cdot$ kg BM $^{-1}\cdot$ h $^{-1}$ ) replacing a fraction of carbohydrate equally stimulates muscle glycogen resynthesis, despite higher circulating insulin (Beelen *et al.*, 2012; van Hall *et al.*, 2000). It can therefore be concluded that further elevation of insulin does not concurrently accelerate muscle glycogen resynthesis during short-term recovery when ample amounts of carbohydrate are provided. Nonetheless, elevating post-exercise insulin levels may accelerate liver glycogen stores (Beelen *et al.*, 2012), albeit more research is warranted to examine the effects of maximising insulin secretion on glucose uptake and glycogen resynthesis in other tissues such as the liver.

The present finding that protein co-ingestion is equally effective in muscle glycogen repletion is consistent with some investigations (Beelen *et al.*, 2012; Betts *et al.*, 2008; Howarth *et al.*, 2009), but this is not without contention (Berardi *et al.*, 2006; Ivy *et al.*, 2002). These divergent findings may relate to a number of factors that are known to influence the rate of muscle glycogen resynthesis; paramount of these is the magnitude of glycogen depletion from the prior exercise bout (Zachwieja *et al.*, 1991). In accordance, it has been shown that muscle glycogen resynthesis occurs in two phases, with the initial insulin-independent phase exhibiting higher muscle glycogen resynthesis rates (Maehlum *et al.*, 1977). However, this insulin-independent phase was shown to occur when glycogen is depleted to low levels below  $150 \text{ mmol}\cdot\text{kg dm}^{-1}$  at the end of an exercise bout (Jentjens and Jeukendrup, 2003; Maehlum *et al.*, 1977). It is therefore noteworthy the muscle glycogen concentrations following an initial exercise bout vary considerably between studies, ranging between  $60 - 259 \text{ mmol}\cdot\text{kg dm}^{-1}$  (Betts and Williams, 2010). In the current experiment muscle glycogen concentrations reached  $\approx 100 \text{ mmol}\cdot\text{kg dm}^{-1}$  at fatigue in Run-1, which may partly explain the augmented ( $\approx 40 \text{ mmol}\cdot\text{kg dm}^{-1}\cdot\text{h}^{-1}$ ) muscle glycogen resynthesis rates relative to the considerably lower ( $< 15 \text{ mmol}\cdot\text{kg dm}^{-1}\cdot\text{h}^{-1}$ ) rates of muscle glycogen resynthesis reported in other running-based investigations where muscle glycogen concentrations ranged between  $160\text{-}235 \text{ mmol}\cdot\text{kg dm}^{-1}$  following the initial non-exhaustive exercise bout (Betts *et al.*, 2008; Lunn *et al.*, 2012). Thus, the present study confirms that maximal rates of muscle glycogen resynthesis during short-term recovery can be achieved following a prior exhaustive running bout.

In summary, the addition of  $0.4 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  protein to a solution providing  $0.8 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$  carbohydrate during a 4 h recovery from prolonged running exercise resulted in a similar rate of muscle glycogen resynthesis as when a carbohydrate solution matched in energy content was ingested. Moreover, the inclusion of protein did not alter muscle glycogen degradation during running exercise subsequent to recovery. The onset of fatigue during both treatments was associated with critically low muscle glycogen concentrations and hence the availability of skeletal muscle glycogen appears to be a major determinant in restoration of exercise capacity following short-term recovery. These data therefore suggest that replacing a fraction of carbohydrate with protein following prior exhaustive running equally elicits high muscle glycogen

resynthesis rates relative to an isocaloric carbohydrate solution. Given that the addition of protein did not impair glycogen storage or restoration of subsequent endurance capacity, it may be prudent to co-ingest protein to maximise muscle glycogen repletion under circumstances where the amount of carbohydrate that can be ingested is limited (i.e.  $< 1 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) or when other aspects of post-exercise recovery nutrition (i.e. muscle protein synthesis and tissue reconditioning) are considered.



## CHAPTER 6

### RELIABILITY OF TIME TO EXHAUSTION TREADMILL RUNNING AS A MEASURE OF HUMAN ENDURANCE CAPACITY

#### Introduction

To examine the effects of different nutritional interventions on performance and fatigue, exercise protocols often require individuals to exercise until the point of volitional exhaustion (time to exhaustion; TTE) or complete a set distance or amount of work as quickly as possible (time trial; TT). A meaningful physiological performance test requires reliability, such that reproducible results are obtained when a test is performed repeatedly (Hopkins *et al.*, 2001). The internal validity of TTE is well established to measure fatigue (Bergstrom *et al.*, 1967; Maughan *et al.*, 1989; Wilson and Maughan, 1992), with external validity also apparent for various exercise scenarios and occupational tasks (Alghannam *et al.*, 2014). However, a focus of much attention and debate is the reliability of TTE in study protocols, particularly relative to TT tests (Currell and Jeukendrup, 2008; Hopkins *et al.*, 2001), evidenced by some investigations reporting insufficient reliability of TTE with a coefficient of variation (CV) of 13-27 % (Jeukendrup *et al.*, 1996; Laursen *et al.*, 2007; McLellan *et al.*, 1995). Nevertheless, it can be postulated that the reliability of any exercise protocol will depend on the specific measures/protocol employed and thus no single CV can be assigned to a given exercise test, which can be expected to vary within and between laboratories.

The mode and intensity of a TTE test appears to influence the degree of variability (McLellan *et al.*, 1995). With longer duration fixed intensity, TTE protocols may carry greater variability than those with shorter duration (Currell and Jeukendrup, 2008; Hopkins *et al.*, 2001). Nonetheless, it has been argued that TTE may not be a reliable measure when the exercise intensity is increased above 80 %  $\dot{V}O_{2\max}$  (Krebs and Powers, 1989), which may be related to differences in fatigue mechanisms and

inter-individual differences once test intensity approximates the aerobic/anaerobic ‘threshold’ (Betts *et al.*, 2005). To support this notion in TTE exercise protocols, it has been shown that exercise intensities  $< 80\% \dot{V}O_{2\max}$  appear to have lower CV ( $\approx 9\%$ ) (Gleser and Vogel, 1971; Maughan *et al.*, 1989) than those utilising maximal short-duration protocols (Billat *et al.*, 1994), but this is not universal (Coggan and Costill, 1984). Little if any information is available that reports the variability in TTE during submaximal treadmill running in endurance-trained individuals. In concordance, the aim of this study was to investigate the test-retest reliability of prolonged moderate- to high-intensity run time to exhaustion as a measure of human endurance capacity.

## Materials and Methods

### Participants

Sixteen healthy active males ( $n=14$ ) and females ( $n=2$ ) participated in this study (Mean  $\pm$  SD; age  $23 \pm 7$  years, body mass (BM),  $70.4 \pm 8.6$  kg, and  $\dot{V}O_{2\max}$   $62 \pm 5.4$  mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$ ). These individuals included  $5 \pm 1$  h per week of endurance running as part of their habitual training. Each individual was fully briefed regarding the nature of the study and provided informed consent prior to participation. The study was approved by National Health Services Research Ethics Committee (Ref: 09/H0101/82) and was part of a wider project on the effects of nutrition on post-exercise recovery and repeated exercise (Alghannam *et al.*, 2014).

### Preliminary measurements

Participants underwent one preliminary visit to determine submaximal ( $\dot{V}O_2$ ) and maximal ( $\dot{V}O_{2\max}$ ) oxygen uptakes (Taylor *et al.*, 1955) on a motorised treadmill (Ergo ELG70, Woodway, Weil am Rhein, Germany) and a second familiarisation visit during which participants underwent the main experimental procedures exactly as per the main experimental arms described below.

### Experimental design

Participants completed two main trials (T1 and T2) in a repeated measures experimental design separated by an interval of three weeks (95 % CI; 2-3 weeks). Each trial involved a run to exhaustion at 70 %  $\dot{V}O_{2\max}$ . A weighed dietary record was completed 48 h preceding the familiarisation trial, and was subsequently repeated prior to the commencement of the main trials ( $11150 \pm 2514 \text{ kJ}\cdot\text{d}^{-1}$ ;  $53 \pm 5 \%$  CHO;  $22 \pm 10 \%$  fat;  $25 \pm 7 \%$  protein). Participants were provided with a standardised meal (3180 kJ; 57 % CHO; 24 % Protein; 19 % fat) to be consumed as their final caloric intake before testing, consumed in the evening ( $12 \pm 1 \text{ h}$ ) before the familiarisation trial and replicated prior to each main trial. Participants also abstained from alcohol consumption and refrained from strenuous physical activity (with any light exercise recorded and matched) during this 48 h period of dietary and lifestyle standardisation between trials.

### Experimental protocol

The experimental protocol is described in detail elsewhere (Alghannam *et al.*, 2014). Briefly, each participant arrived to the laboratory  $\approx 0800 \text{ h}$  in a post-absorptive state following  $\geq 10 \text{ h}$  overnight fast. After confirming their informed consent to take part in the study, each participant provided a urine sample to assess hydration via cryoscopic osmometer (Advanced Instruments, Inc, Norwood, MA, USA) and adequate hydration was assumed for osmolality values  $\leq 900 \text{ mOsm}\cdot\text{kg}^{-1}$  (Shirreffs and Maughan, 1998) and then nude BM was recorded (Weylux 424, Fereday & Sons Ltd., UK) to the nearest 0.1 kg. A standardised 5-min warm-up at 60 %  $\dot{V}O_{2\max}$  was used before running at a speed equivalent to 70 %  $\dot{V}O_{2\max}$  until the point of volitional exhaustion. Once participants indicated that they were unable to sustain the exercise intensity, the prescribed running speed was reduced to walking (walk-1;  $4.4 \text{ km}\cdot\text{h}^{-1}$ ) for 2-min intervals. This procedure was repeated for a second time (walk-2) and only on the third occasion when participants indicated they could no longer sustain the prescribed exercise intensity was fatigue accepted and time to exhaustion (TTE) recorded. The time during walking was excluded from the total TTE. Average one-minute hearts rates (Polar FT2, Kempele, Finland), and ratings of perceived exertion

(RPE; (Borg, 1973)) were taken at 30 min intervals during the runs. Water intake was permitted *ad libitum* during the familiarisation trial and then matched for subsequent trials ( $0.4 \pm 0.3$  litres). Nude BM was then recorded to assess hydration status through percentage change in mass. Ambient temperature and humidity were recorded at 60 min intervals throughout the trials using a portable weather station (WS 6730; Technoline, Berlin, Germany). Participants exhibited adequate degrees of pre-exercise hydration status among experimental conditions ( $p = 0.7$ ), with mean urine osmolality values of  $533 \pm 298$  and  $506 \pm 266$  mOsm.kg<sup>-1</sup> in T1 and T2, respectively. Similarly, the sweat loss during the exhaustive run was not significantly different ( $p = 0.7$ ) across T1 and T2, as reflected by  $-1.8 \pm 0.8$  % and  $-1.7 \pm 0.8$  %, respectively. Environmental conditions were standardised between the experimental trials, with barometric pressure ( $741 \pm 9$  and  $741 \pm 9$  mmHg;  $p = 0.7$ ), ambient temperature ( $19.8 \pm 0.9$  and  $19.8 \pm 0.9$  °C;  $p = 0.9$ ) and humidity not statistically different ( $47 \pm 7$  and  $47 \pm 8$  %;  $p = 0.9$ ) between T1 and T2, respectively. Background music and verbal encouragement was standardised between trials (Atkinson *et al.*, 2004) and participants were unaware of the time elapsed during the exercise capacity test.

### Statistical analysis

Paired differences were tested for normality using the Shapiro-Wilk test and single comparisons between two means were analysed by using a paired sample *t*-tests when normally distributed. Where data were deemed in violation of normality, a non-parametric equivalent (i.e. Wilcoxon signed rank test) was employed to compare medians. The mean coefficient of variation (standard deviation/mean x 100) with the associated 95 % confidence intervals (CI) was calculated to establish error in measurement between TTE protocols. Additionally, intra-class correlation coefficients (ICC) with a two-way random effects 95 % CI were used to determine test-retest reliability, with  $\leq 0.50$  indicating moderate reliability, 0.70-0.89 high reliability, and  $\geq 0.90$  very high reliability (Dupuy *et al.*, 2012). Pearson product moment correlation coefficient (*r*) and coefficient of determination ( $R^2$ ) were conducted to assess the association between the performed exercise protocols. Bland and Altman plots were used to determine absolute bias using 95 % limits of agreement (LoA). A two-way linear mixed model was used to identify differences between trials over time. Statistical procedures were performed using commercially

available software (IBM SPSS version 21.0, SPSS Inc., Chicago, IL) and significance was set at an alpha level  $\leq 0.05$ . Unless otherwise stated, all results were reported as the mean  $\pm$  standard deviation (SD) of the mean.

## Results

There was no systematic bias in time to exhaustion between T1 and T2, and neither were any trial order differences apparent at the relative time points of walk-1 ( $84 \pm 19$  *versus*  $90 \pm 29$  min), walk-2 ( $91 \pm 19$  *versus*  $96 \pm 29$  min) or TTE ( $96 \pm 19$  *versus*  $101 \pm 29$  min), respectively (Figure 6.1). The mean  $\pm$  95 % CI of the typical error of measurement expressed as CV was 5.4 % (1.4 – 9.6) at the point of exhaustion, hence was marginally lower than walk-1 (6.3 %; 1.7 – 11.0) and walk-2 (5.7 %; 1.4 – 10). Percent change in mean TTE  $\pm$  95 % CI across T1 and T2 trials was 3.7 % (-4 – 11.3), which was also lower than percent change in mean to reach walk-1 (5.7 %; -3 – 15) and walk-2 (4.4 %; -4 – 13). In relation to differences from walk-1 to the point of volitional exhaustion (TTE), no trial  $\times$  time interactions were identified ( $p = 0.8$ ). Although not statistically significant, a trend for effect of time across both trials from the walk-1 until TTE was shown ( $p = 0.07$ ), whereby participants were able to run  $11 \pm 3$  min from walk-1 until the point of volitional exhaustion. Participants were able to run for  $6 \pm 2$  min from walk-1 to walk-2 and  $5 \pm 2$  min from walk-2 to TTE.

Relative exercise intensities were successfully matched between trials and averaged  $69.6 \pm 4.1$  %  $\dot{V}O_{2\max}$  in T1 and  $69.2 \pm 3.8$  %  $\dot{V}O_{2\max}$  in T2. This was additionally verified by the overall heart rate ( $178 \pm 8$  and  $175 \pm 7$  beats $\cdot$ min $^{-1}$ ) and RPE ( $17 \pm 3$  and  $16 \pm 3$ ) measurements in T1 and T2, respectively (Table 6.1). The respiratory exchange ratio using indirect calorimetry was similar during exercise between T1 ( $0.87 \pm 0.1$ ) and T2 ( $0.89 \pm 0.1$ ). No time $\times$ trial interactions were observed in whole-body carbohydrate oxidation ( $F = 0.035$ ;  $p = 0.85$ ) or fat oxidation ( $F = 0.006$ ;  $p = 0.94$ ). During exercise, whole-body carbohydrate oxidation was  $2.1 \pm 0.4$  g $\cdot$ min $^{-1}$  and  $2.3 \pm 0.6$  g $\cdot$ min $^{-1}$ , while fat oxidation was  $0.6 \pm 0.2$  g $\cdot$ min $^{-1}$  and  $0.6 \pm 0.2$  g $\cdot$ min $^{-1}$  in T1 and T2, respectively (Table 6.2). Thus, no systematic bias was observed in exercise intensity, HR, RPE or substrate metabolism across trials ( $p > 0.05$ ).

The average intra-class correlation coefficient ( $\pm 95\%$  CI) revealed high reliability of 0.88 (0.67 – 0.96) at the time of exhaustion between the trials. A similar pattern was shown at walk-1 and walk-2 with ICC of 0.86 (0.62 - 0.95) and 0.87 (0.65 – 0.96), respectively. A high correlation coefficient ( $r = 0.86$ ; 0.62 – 0.95) was observed between the two TTE exercise protocols ( $p < 0.001$ ; Figure 6.2), as evidenced by the coefficient of determination ( $R^2 = 0.73$ ). The correlation coefficient and coefficient of determination were similarly high during walk-1 ( $r = 0.84$  (0.59 – 0.94);  $R^2 = 0.70$ ) and walk-2 ( $r = 0.85$  (0.62 – 0.95);  $R^2 = 0.73$ ). Figure 6.3 illustrates the difference between trials through the use of Bland and Altman plots displaying the absolute bias and 95 % LoA. The absolute bias  $\pm 95\%$  LoA was  $4 \pm 31$  minutes.

**Table 6.1** Percent (%)  $\dot{V}O_{2\max}$ , RPE and heart rate responses to the first and second run times to exhaustion.

	Pre	30 min	60 min	90 min	TTE
<b>% <math>\dot{V}O_{2\max}</math></b>					
Trial 1	—	67.7 ± 5.6	69.1 ± 4.6	71.5 ± 1.7	70.0 ± 4.4
Trial 2	—	67.5 ± 3.4	68.3 ± 3.9	70.8 ± 1.9	70.3 ± 5.9
<b>RPE (6-20)</b>					
Trial 1	—	13 ± 1	16 ± 2	18 ± 1	20 ± 0
Trial 2	—	13 ± 2	15 ± 2	17 ± 2	20 ± 0
<b>Heart rate (beats·min<sup>-1</sup>)</b>					
Trial 1	72 ± 13	172 ± 8	179 ± 9	181 ± 10	181 ± 9
Trial 2	72 ± 12	169 ± 6	175 ± 7	178 ± 9	178 ± 9

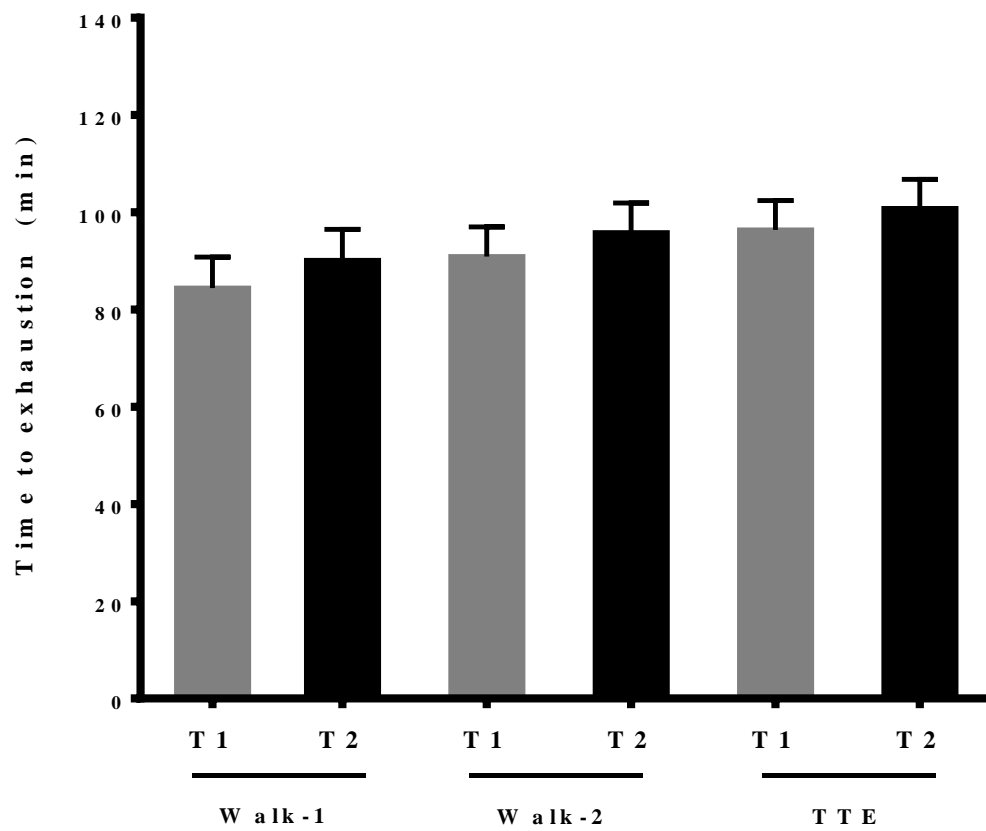
Data are mean ± SD. TTE; time to exhaustion.

**Table 6.2** Substrate metabolism and respiratory exchange ratio (RER) in the first and second run times to exhaustion.

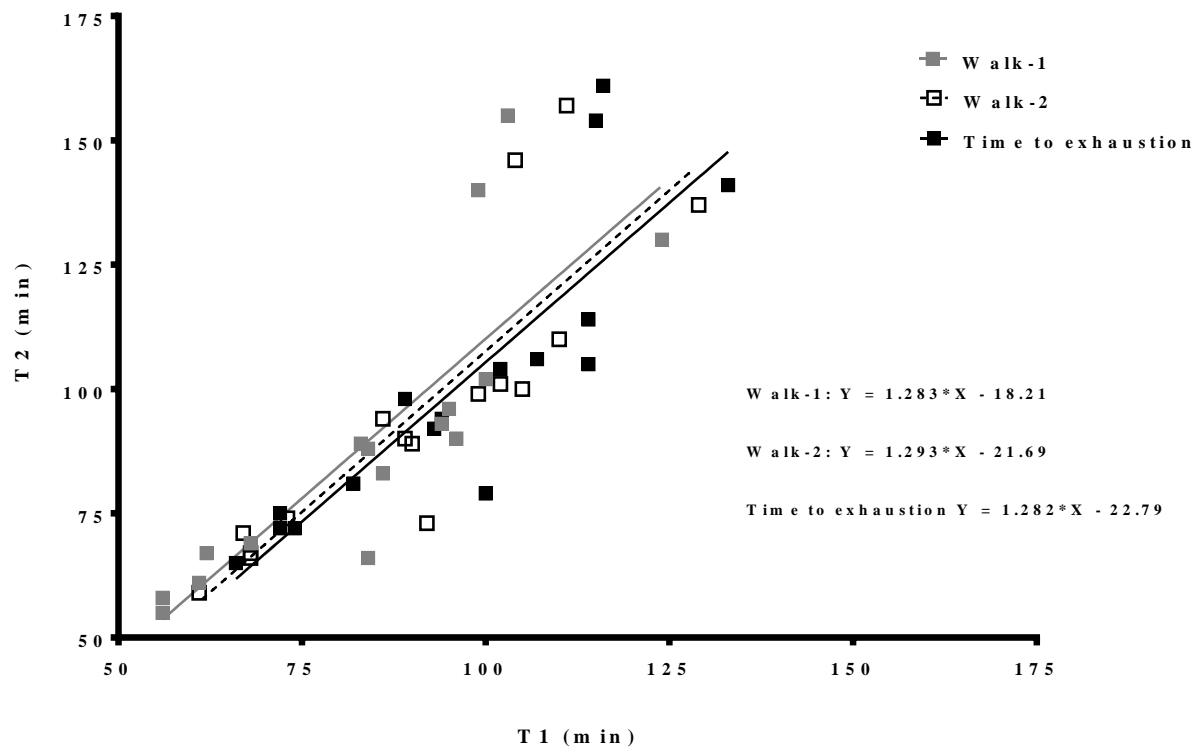
	Run-1				
	Pre	30 min	60 min	90 min	TTE
<b>Carbohydrate oxidation (<math>\text{g}\cdot\text{min}^{-1}</math>)</b>					
Trial 1	0.22±0.14	2.10±0.42	2.12±0.58	2.04±0.28	2.06±0.77
Trial 2	0.29±0.17	2.35±0.69	2.30±0.63	2.32±0.39	2.21±0.83
<b>Lipid oxidation (<math>\text{g}\cdot\text{min}^{-1}</math>)</b>					
Trial 1	0.06±0.06	0.54±0.22	0.61±0.20	0.72±0.23	0.73±0.32
Trial 2	0.05±0.06	0.46±0.21	0.56±0.24	0.66±0.45	0.70±0.36
<b>RER</b>					
Trial 1	0.84±0.11	0.88±0.04	0.88±0.04	0.86±0.04	0.87±0.07
Trial 2	0.84±0.09	0.90±0.04	0.89±0.05	0.88±0.08	0.88±0.07

Data are mean  $\pm$  SD. TTE; time to exhaustion.

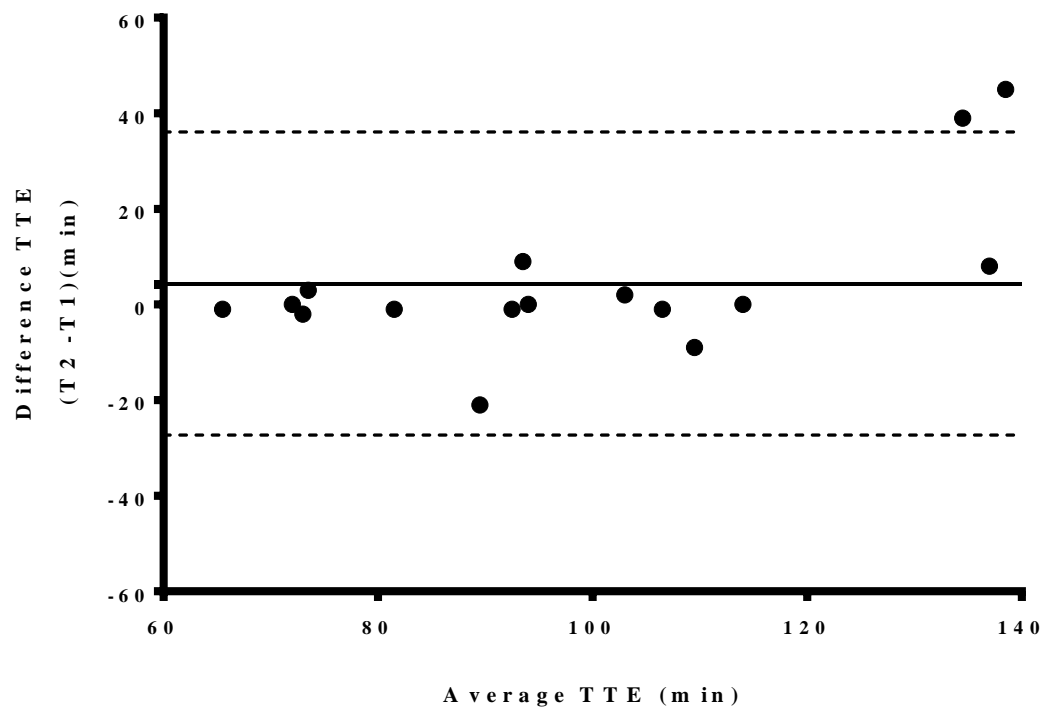




**Figure 6.1** Run times compared between trials during walk-1, walk-2 and time to exhaustion. Data are means  $\pm$  CI. TTE, time to exhaustion; T1, trial 1; T2, trial 2.



**Figure 6.2** Scatterplot showing the relationships between the two run times to exhaustion. Straight lines represent best fit for walk-1, walk-2 and time to exhaustion. T1, trial 1; T2, trial 2.



**Figure 6.3** Bland-Altman plot of absolute agreement between two run times to exhaustion. TTE, time to exhaustion; T1, trial 1; T2, trial 2.

## Discussion

This study investigated the test-retest reliability of prolonged moderate- to high-intensity run time to exhaustion as a measure of human endurance capacity. Here we demonstrate high relative and absolute test-retest reliability of time to exhaustion as measure of endurance capacity during prolonged (> 60 min) treadmill running and thus may be acceptable to detect small but meaningful effects upon endurance capacity. To our knowledge, this is the first study to assess the reliability of a treadmill based prolonged exhaustive exercise protocol.

Previous comparisons between TT and TTE have examined the reliability of these measures, and notable variability in the reliability of TTE exercise protocols can be observed relative to TT measures (Currell and Jeukendrup, 2008; Hopkins *et al.*, 2001). However, numerous extraneous variables may affect reliability such as the mode of exercise employed, individuals being tested, laboratory environment and, importantly, familiarisation. The low measurement error presented here is in agreement with a study using prolonged (> 60 min) cycling (Maughan *et al.*, 1989) but is in contrast to other prolonged cycling-based investigations (Gleser and Vogel, 1971; Jeukendrup *et al.*, 1996). Moreover, the present findings are not consistent with other shorter duration running-based TTE protocols of only 6-18 min (Billat *et al.*, 1994; Laursen *et al.*, 2007). Gleser and Vogel (1971) have reported the presence of a systematic order effect and an associated higher degree of variability (CV= 13 %) when repeated TTE exercise bouts were performed in untrained participants unaccustomed to exercise tests to exhaustion and were not familiarised to the exercise protocol (Gleser and Vogel, 1971). This was further corroborated by McLellan *et al.* (1995), who reported a CV of 17 % in participants who were not aerobically trained (McLellan *et al.*, 1995). Indeed, TT may carry greater reliability from the outset, particularly if using relevant athletes and/or if repetitive familiarisation with TTE is to be avoided. However, the current findings further support the notion that the reliability of an exercise protocol may vary substantially between laboratories and the degree of variability in TTE tests can be influenced by familiarisation, participant characteristics and the specific protocol utilised. In the current study, young, healthy and aerobically trained participants underwent a familiarisation trial identical to the experimental procedures. These considerations may have a profound impact in the sensitivity of an exercise protocol (Gleser and Vogel, 1971; McLellan *et al.*, 1995). Critically, no training effect would therefore be expected nor was detected in our cohort of participants, as evidenced by the similar relative exercise intensity, RPE and HR response

between trials. In addition to the aforementioned steps, we also employed a number of measures to increase the absolute reliability of the TTE protocol. We standardised the nutritional status of participants prior to the commencement of each trial to diminish any carry over effect to metabolism and/or performance (Braun and Brooks, 2008). Indeed, it has been postulated that longer duration protocols may present higher variation than short protocols (Currell and Jeukendrup, 2008), mainly associated with the known role of prior nutrition and the inherent link between pre-exercise glycogen availability and the endurance capacity (Bergstrom *et al.*, 1967).

With regards to the prolonged nature of the adopted TTE protocol, it is also conceivable that environmental conditions and hydration status may influence the reliability of the tests. In the present study, the laboratory environment was successfully standardised as shown in the similar ambient conditions (temperature, humidity and barometric pressure), pre-trial hydration status and changes in BM across the experimental procedures. Collectively, the control measures conducted in the present study may have contributed to the minimised absolute measurement error (CV= 5.4 %) associated with previously reported TTE protocols (Billat *et al.*, 1994; Laursen *et al.*, 2007).

An important quality of a valid test of prolonged endurance capacity is that participants reach a ‘metabolic endpoint’ (i.e. volitional exhaustion coincident with metabolic substrate disturbance/depletion as opposed to injury, stomach discomfort or mere boredom). To help participants gauge their fatigue more accurately, we incorporated two 2-min walks in the current protocol before terminating the TTE test. Prior to each trial, participants were informed not to use any of the walks for tactical reasons but rather to indicate the need to reduce the running speed only once they felt unable to sustain the exercise intensity (i.e. where they would stop if there were to be no further opportunity to continue). These individuals were able to run for  $11 \pm 3$  min from walk-1 to TTE, which represents  $\approx 11$  % of the total time to exhaustion during the endurance capacity test. Muscle glycogen resynthesis would be restored at very low rates in the absence of carbohydrate ingestion ( $0.5 \text{ mmol} \cdot \text{kg dry mass}^{-1} \cdot \text{min}^{-1}$ ; (Ivy *et al.*, 1988a)). Thus, given that muscle glycogen utilisation would be  $3.2 \text{ mmol} \cdot \text{kg dry mass}^{-1} \cdot \text{min}^{-1}$  during treadmill running the intensity employed here (Tsintzas *et al.*, 1996b), any resynthesis of muscle glycogen during the 4-min of walking could only

account for < 1 min of extended exercise time. It is therefore reasonable to suggest that including walking intervals is important to help participants better gauge their level of fatigue and more completely deplete their muscle glycogen levels.

There were several important limitations in the current study. The experiment was not designed to assess the specific role of individual extraneous variables in improving reliability of TTE exercise protocol. As a consequence, it is not possible to determine the precise factor(s) contributing to the lower variability reported here than previously. It is also noteworthy that expired gas and blood sampling measurements were obtained during the TTE protocol (blood data not shown), which may have had a negative influence on the mental concentration of participants (Jeukendrup *et al.*, 1996), albeit that is the context in which TTE tests are normally used. It is also worth stating that only two female participants took part in the current study and thus sex-specific difference on the variability of TTE exercise protocols remain largely unknown. Notwithstanding that, the variability between the two trials in these females (CV= 1.5 %) reflects a similar pattern to their male counterparts; a larger sample of female participants is required to establish the presence of any sex-specific differences on variability of TTE measures.

Reliability could be considered as the amount of measurement error that has been deemed acceptable for the effective practical use of a measurement tool (Atkinson and Nevill, 1998). In this respect, it may be considered that TT and TTE are inherently different measurement tools, each encompassing their own level of acceptable 'noise'. Indeed, it has been postulated that the signal-to-noise ratio may be greater in TTE when compared to TT (Hopkins *et al.*, 2001). Accordingly, a reliable test can be judged on the basis of its sensitivity for detecting worthwhile changes (Laursen *et al.*, 2007). The bias present between the two TTE tests was shown to be 4 % and in accordance can be deemed as acceptable measurement tool under the commonly accepted measurement error of  $\leq 5$  % (Atkinson, 2003). This is further supported when considering a greater signal-to-noise ratio (i.e. relative higher variability than TT but larger changes in the outcome measure) as demonstrated by > 20 % change typically seen in TTE in response to various experimental interventions (Galloway and Maughan, 2000; Maughan *et al.*, 1996; Maughan *et al.*, 1989), despite the fact that the magnitude of change is likely to be dependent on the specific intervention. Therefore, when considering the

culmination of the relative and absolute reliability measurements observed in the current study, it would appear that the current TTE protocol can be a reliable tool for research purposes.

In conclusion, this study demonstrates high absolute and relative reliability between two exhaustive bouts of prolonged moderate- to high-intensity treadmill running in endurance trained participants. The use of aerobically trained individuals who are familiarised to TTE exercise protocol may have contributed in obtaining more reliable measurement outcomes (Hopkins *et al.*, 2001). Moreover, the inclusion of short intervals of reduced intensity during the exercise protocol may be an important consideration to reach a ‘metabolic endpoint’ and therefore a more accurate reflection of endurance capacity.

## CHAPTER 7

### IMPACT OF POST-EXERCISE PROTEIN INGESTION ON ENDURANCE TRAINING ADAPTATION

#### Introduction

The potential for nutrition to influence exercise training-induced adaptations has long been recognised. Nutrient availability affects the acute exercise response and, when applied repeatedly, may modulate chronic adaptations in response to training (Hawley *et al.*, 2011). Exercise and protein ingestion both have an effect on muscle protein synthesis, with the consequent subtle changes in muscle quantity and/or quality culminating in training adaptations if sustained over weeks or months (Betts and Williams, 2010; Hawley *et al.*, 2011). Thus, nutritional strategies provided in close temporal proximity to exercise have the potential to optimise training adaptations by taking advantage of an adaptive environment created by exercise (Hawley *et al.*, 2006; Miller, 2007). While a multitude of investigations have been conducted in an acute laboratory-based setting (Betts and Williams, 2010), any effect of protein ingestion may be more likely beyond the acute post-exercise recovery phase via the accumulation of acute responses to repetitive exercise plus appropriate feeding (Atherton and Smith, 2012; Phillips, 2012). The latter would seem logical given that the mechanisms underpinning adaptation to exercise involve gradual alterations in protein content and enzyme activity, which may take weeks/months to reach a new functional threshold (Egan and Zierath, 2013).

Recent training interventions have demonstrated the role of protein ingestion in improving/maintaining: exercise performance; tolerance to intensified periods of training; and the magnitude of training adaptations when repeated bouts of exercise are performed (Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b; Walker *et al.*, 2010; Witard *et al.*, 2011). Despite these potential benefits, only a few studies examined the role of post-exercise protein feeding in facilitating the adaptive responses to endurance exercise training (Cramer *et al.*, 2012; Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b; Robinson *et al.*, 2011). However, the inclusion of other nutrients such as caffeine, flavonoids and multivitamins



(Cramer *et al.*, 2012; Ferguson-Stegall *et al.*, 2011a), in addition to the absence of macronutrient-specific comparisons by not including a carbohydrate-only control (Okazaki *et al.*, 2009b), makes it difficult to distinguish the distinct role of protein in mediating improved training adaptation. Thus, how post-exercise carbohydrate-protein supplementation can influence the adaptive response to long term (i.e. six weeks) running-based endurance training relative to an energy matched carbohydrate supplement have not been determined.

Although it has been established that protein ingestion following endurance exercise increases mixed muscle protein synthesis (Harber *et al.*, 2010; Howarth *et al.*, 2009; Lunn *et al.*, 2012), little change in mitochondrial protein synthetic response occurs during short-term recovery from exercise (Breen *et al.*, 2011; Coffey *et al.*, 2011). Thus, it is possible that post-exercise protein feeding enhances the adaptive response to endurance training through other mechanisms. Namely, cardiovascular improvements in maximal oxygen uptake by an increase in plasma albumin content and plasma volume expansion have been reported when protein was ingested following exercise (Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b), without any differences in the magnitude of improvements in intramuscular adaptive markers (Ferguson-Stegall *et al.*, 2011a). Interestingly, post-exercise ingestion of both isolated whey protein and milk-based protein in the form of chocolate milk potentiated improvements in maximal oxygen uptake during cycling-based endurance training relative to carbohydrate and/or placebo (Ferguson-Stegall *et al.*, 2011a; Okazaki *et al.*, 2009b). Collectively, these findings may infer nutrient-specific effects of post-exercise supplementation on amplifying cardiovascular adaptation secondary to endurance exercise training.

Therefore, the aim of this study was to examine the effects of post-exercise protein ingestion on the magnitude of exercise-induced cardiovascular and intramuscular adaptations to six weeks of endurance training. It was hypothesised that protein co-ingestion would increase the magnitude of cardiovascular training adaptations secondary to an increase in plasma albumin content and an expansion in plasma volume.

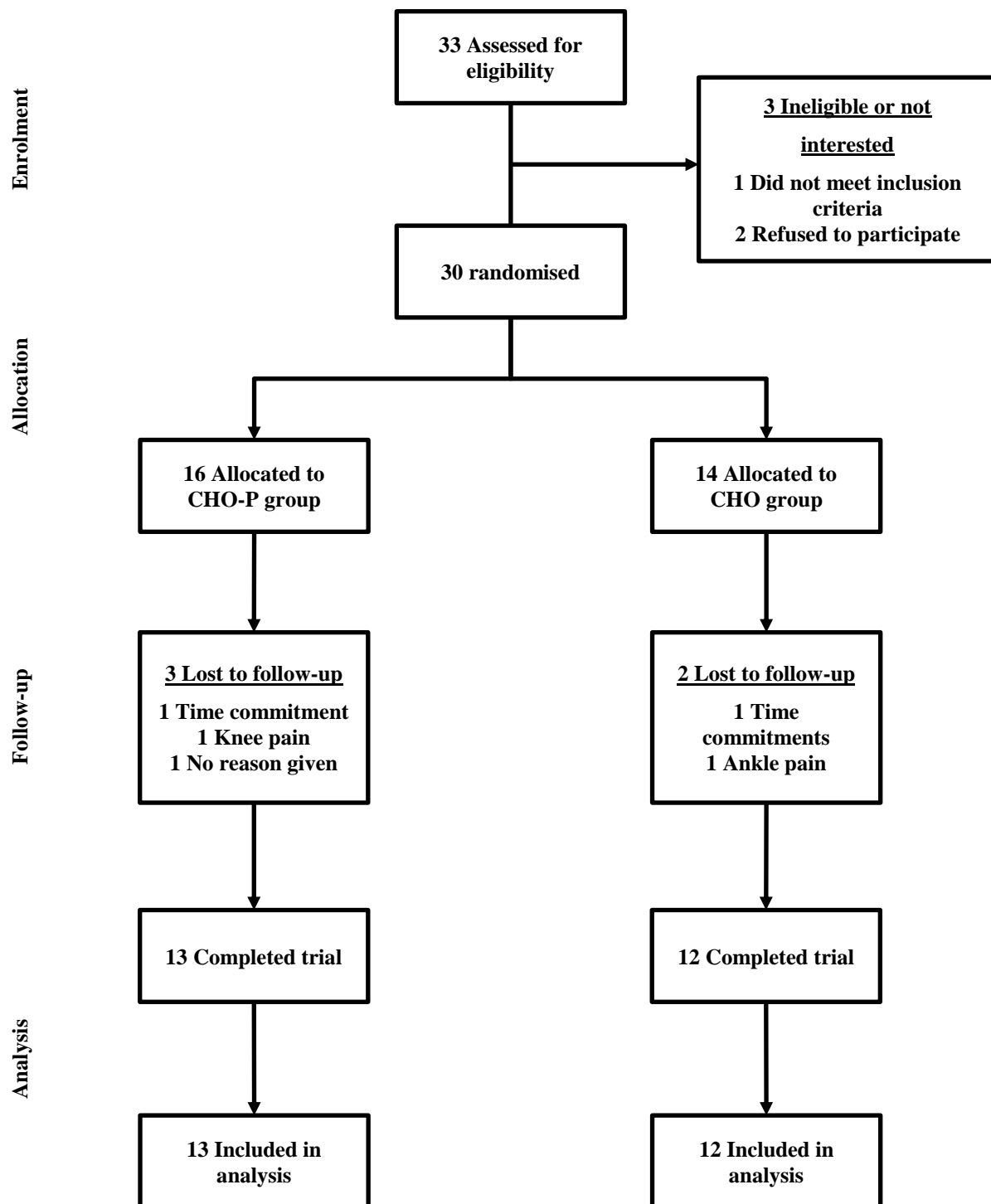
## Materials and methods

### Approach to research question

The primary outcome of this trial was to explore cardiovascular (i.e. changes in maximal oxygen uptake ( $\dot{V}O_{2\max}$ ), plasma albumin content and changes in plasma volume) and intramuscular (i.e. selected genes involved in cellular adaptive processes related to exercise/nutrition) endurance training adaptations and whether protein co-ingestion improves this response. A carbohydrate-protein mixture was evaluated relative to an energy-matched carbohydrate control. Given that the target population were not previously trained and subjected to endurance training, and that reversal of these training-induced adaptations are relatively slow in such individuals (Mujika and Padilla, 2001), the study adopted an independent-measures parallel group design with a relatively larger cohort of participants to account for inter-individual variability. The duration of the training period was six weeks. This duration was deemed adequate based on previous literature, which demonstrated that approximately four weeks (total number of sessions was 20-22 during the entire protocol) is sufficient to elicit meaningful changes in maximal oxygen uptake ( $\dot{V}O_{2\max}$ ) by 6-14 % and oxidative enzyme activity by 35-50 % in untrained and moderately trained individuals (Ferguson-Stegall *et al.*, 2011a; Liu *et al.*, 2012).

### Experimental design

In a randomised investigator-participant double-blind parallel group design, participants were randomly assigned to a group receiving a supplement containing carbohydrate (CHO trial) or a carbohydrate plus protein (CHO-P trial) ingested immediately post-exercise and 1 h after each training session. Participants were required to undergo six weeks of treadmill-based endurance training. The entire protocol was eight weeks and comprised: i) a baseline testing week with two exercise sessions; ii) the first, second and third weeks of training at 70 % ( $\dot{V}O_{2\max}$ ); iii) the fourth, fifth and sixth weeks of training at 75 %  $\dot{V}O_{2\max}$ ; and iv) a final week for post-intervention testing. A total of 26 training sessions were prescribed for each participant (4 sessions per week).



**Figure 7.1** Flow diagram of the study.

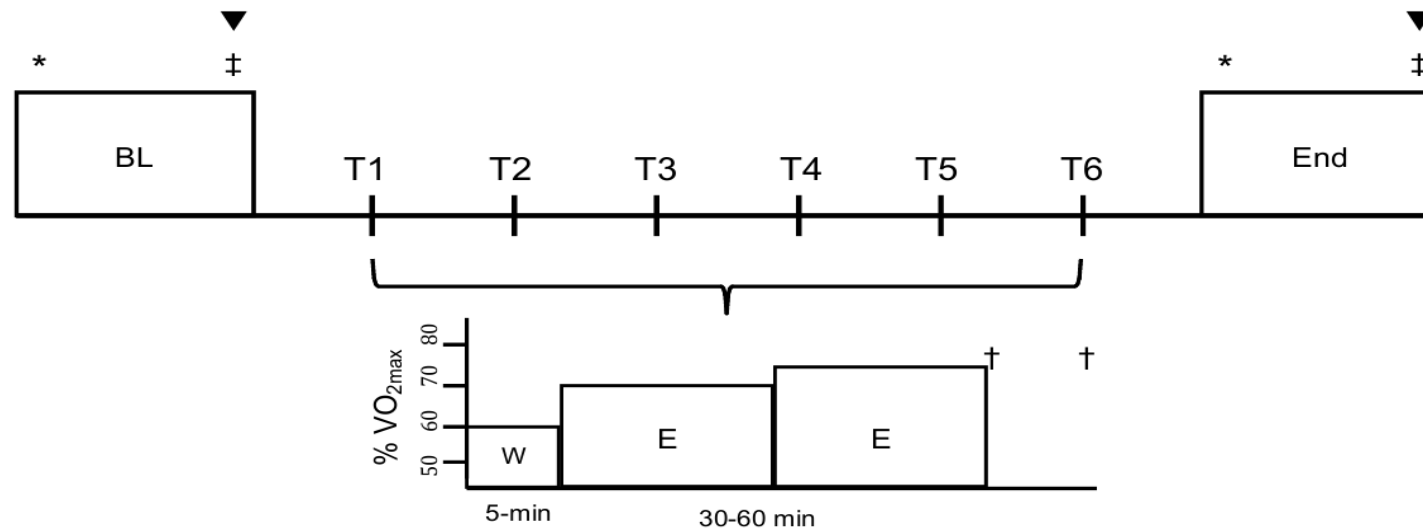


Figure 7.2. Schematic representation of the experimental protocol. \*,  $\dot{V}O_{2max}$ ; †, Expired gas and blood sample; ▼, muscle biopsy; BL, baseline testing; End, follow-up testing; T, training week; W, warm-up; E, exercise session; †, Supplement ingestion (immediately postexercise and 1 h later).

**Participants**

Twenty-three healthy men and two healthy women participated in the study. Randomisation procedures stratified for sex to ensure equal distribution between treatments. The procedures and potential risks were explained to all participants both verbally and in writing prior to obtaining their written informed consent to take part, and administration of medical health questionnaires to ensure the absence of any risks associated with the nature of the study. This study was approved by the National Health Service (NHS) South West 3 Research Ethics Committee (13/SW/0239) and subsequently registered as a controlled trial (ISRCTN27312291).

**Nutritional supplements**

Participants in the CHO group received 1.6 grams per kilogram of body mass ( $\text{g} \cdot \text{kg BM}^{-1}$ ) of carbohydrate (sucrose), while the CHO-P received a carbohydrate (sucrose) plus protein (whey protein hydrolysate) supplement at an ingestion rate of  $0.8 \text{ g} \cdot \text{kg BM}^{-1}$  and  $0.8 \text{ g} \cdot \text{kg BM}^{-1}$ , respectively. All supplements were provided in a sachet form and instructions for solution preparation were provided to participants to achieve a volume for ingestion of  $10 \text{ ml} \cdot \text{kg BM}^{-1}$ . The estimated amount of energy available for metabolism for both trials was therefore  $26.8 \text{ kJ} \cdot \text{kg BM}^{-1}$  (Table 7.1).

**Table 7.1** Nutritional composition of the supplements.

	<b>CHO trial</b>	<b>CHO-P trial</b>
<b>Sucrose (g·l)</b>	80	40
<b>Lactose (g·l)</b>	–	$\leq 2.3^*$
<b>Protein (g·l)</b>	–	40
<b>Fat (g·l)</b>	–	$\leq 2.4^*$
<b>Sodium (g·l)</b>	–	0.72
<b>Potassium (g·l)</b>	–	0.10
<b>Calcium (g·l)</b>	–	0.15
<b>Magnesium (g·l)</b>	–	0.01
<b>Phosphorous (g·l)</b>	–	0.19
<b>Chloride (g·l)</b>	–	0.29
<b>Energy (kJ·l)</b>	1339	1339

\*= Assay unable to detect values below this number, so the caloric content for fat and lactose was assumed negligible.

### Baseline testing

Prior to the start of the training period, participants attended the laboratory on two occasions. The first visit included anthropometric assessment of stature and body mass. Participants also underwent an assessment of their running economy (i.e.  $\dot{V}O_2 \cdot \text{km}^{-1}$ ) and  $\dot{V}O_{2\text{max}}$  (details provided below). The second preliminary visit was arranged within seven days following the first laboratory visit. Forty-eight hour standardisation of lifestyle was employed (discussed below). This visit involved obtaining fat mass percentage using Bioelectrical Impedance Analysis (BIA) followed by resting expired gas samples to measure resting metabolic rate (RMR). Prior to arrival to the laboratory, participants were instructed to ingest 500 ml of water. After resting on a semi-supine bed for 5 min, two x 5-min baseline resting expired gas samples were taken for the estimation of RMR along with resting heart rate via short-range telemetry (Polar FT2, Kempele, Finland). A 10 ml venous blood sample was also drawn through venepuncture from an antecubital vein to measure different plasma metabolites in addition to plasma volume change. Subsequently, an 80-100 mg muscle biopsy sample was obtained using the needle biopsy technique from the *vastus lateralis*. In relation to eumenorrheic female participants, all measurements were conducted at least three and at most 10 days after the onset of menses (i.e. follicular phase) to ensure low levels of circulating fertility hormones to minimise any measurement errors associated with menstrual cycle (Solomon *et al.*, 1982). Two exercise sessions were then employed during baseline testing, which involved participants running on a motorised treadmill. These were aimed to allow participants to gauge the relative intensity required during their prescribed training intervention, while also familiarising them with treadmill running before the commencement of the six weeks training intervention. Ambient temperature, humidity and barometric pressure were monitored and recorded throughout the trials using a portable weather station (WS 6730; Technoline, Germany). The latter were used to record atmospheric pressure to allow for corrections to standard volumes during expired gas analysis.

### Training intervention

Exercise sessions performed during the baseline testing period were set for 30 min and thereafter the duration of sessions were progressively increased to 40, 50 and 60 min in week 1, weeks 2-3, and weeks 4-6, respectively. Prior to any exercise session, a 5 min warm-up at 60 %  $\dot{V}O_{2\text{max}}$  was performed, followed by treadmill running at a speed corresponding to 70 %

$\dot{V}O_{2\max}$  for the durations indicated above. During the midpoint (training week 3), the speed was increased to elicit 75 %  $\dot{V}O_{2\max}$ . Only water consumption was permitted during exercise sessions and was consumed *ad libitum*. Upon cessation of each exercise session, a post-exercise nutritional supplement was ingested immediately, whilst a second bolus was consumed 1 h after the end of exercise. Once every fortnight, participants reported to the laboratory to provide the nutritional supplements for the subsequent two week training block, with a total of three scheduled meetings throughout the training intervention. This visit was also to confirm the adherence of participants to the prescribed training and supplementation procedures.

### **Follow-up testing**

The follow-up procedures after the training intervention were identical to those in the baseline testing phase of the experiment. Importantly, the interval between the final session of prescribed training and follow-up measurements was kept as close as possible to avoid any de-training effects (Mujika and Padilla, 2001) but while allowing for wash-out of acute carry-over effects from the final exercise session (Thompson *et al.*, 2010). Namely, measurements of running economy and maximal oxygen uptake were taken at least one and at most two days following the final exercise training session. This was then followed by 48 h of standardisation of lifestyle (replicating procedures of dietary records and activity obtained two days before baseline measurements) before obtaining any measurement pertaining to the second follow-up laboratory visit, a period of sufficient length to separate the chronic effects of training from exercise-induced responses (Taylor *et al.*, 1987).

### **Anthropometry**

Body fat percentage (% BF) estimates were determined during the second laboratory visit at baseline and follow-up testing, each following 48 h of standardised lifestyle control. These measurements were based on BIA analysis method (BC-543, Tanita, Tokyo, Japan). This method was shown to overestimate % BF by 3-4 % in healthy lean males and females (< 15 % and < 25 % BF, respectively) and underestimate % BF by 3-4 % in overweight/obese males and females (> 25 % and > 33 % BF, respectively) in a healthy population relative to dual-energy X-ray absorptiometry reference method (Sun *et al.*, 2005). While changes in % BF



were not anticipated in the current study protocol based on findings from similar investigations (Cramer *et al.*, 2012; Ferguson-Stegall *et al.*, 2011a), the bias in BIA relative to dual-energy X-ray absorptiometry measurement was corrected when interpreting any data related to % BF in the current study (Sun *et al.*, 2005).

### **Sub-maximal and maximal oxygen uptake measurements**

Running economy (i.e.  $\dot{V}O_2 \cdot \text{km}^{-1}$ ) and maximal oxygen uptake ( $\dot{V}O_{2\text{max}}$ ) measurements were assessed during the first laboratory visit at baseline and follow-up testing (Taylor *et al.*, 1955) according to the procedures described in section 3.6. These tests initially required participants to run on a motorised treadmill (Ergo ELG70, Woodway, Weil am Rhein, Germany). The data acquired from these tests were subsequently used to calculate the treadmill speeds used during the trial procedures (i.e. speeds that elicit 60, 70 and 75 %  $\dot{V}O_{2\text{max}}$ ) by linear regression (Excel 2010, Microsoft, Redmond, WA, USA).

### **Hydration**

During baseline and follow-up measurements, urine samples were collected to determine hydration via freezing point depression method using a cryoscopic osmometer (Advanced Instruments, Inc, Norwood, MA, USA). The threshold for adequate hydration was assumed for osmolality values  $\leq 900 \text{ mOsm} \cdot \text{kg}^{-1}$  (Shirreffs and Maughan, 1998).

### **Expired gas sampling**

The Douglas bag method (Hans Rudolph, Shawnee, KS, USA) was used for expired gas analysis. The collected gas samples were analysed for relative expired fractions of oxygen and carbon dioxide using a paramagnetic and infra-red analysers, respectively (Servomex, Crowborough, UK). The total volume of expired gas within the Douglas bag was subsequently measured by a dry gas meter (Harvard Apparatus, Kent, UK), with the temperature of expired gases being collected at the time of evacuation by a thermistor probe. Indirect calorimetry based on calculations of oxygen consumption and carbon dioxide production from each bag was then used to obtain RMR. The procedures undertaken by individuals being measured and those applied by the researchers followed recommendations

by The American Dietetic Association related to best practice for measuring RMR (Compher *et al.*, 2006). These procedures included participants arriving from 8-10 h overnight fast following two days of standardised physical activity and diet in a quiet, private space with temperature controlled between 20 and 25°C. Additionally, participants were rested in the same reclined position for 5-7 min before steady-state conditions and measurement interval (two x 5 min periods) occurred.

### **Blood sampling**

A 10 ml blood sample was collected before being dispensed into two x 5 ml EDTA-treated tubes (Sarstedt, Leicester, UK). The first of these tubes was immediately analysed for haemoglobin (Sysmex SF-3000 Sysmex Ltd., Wymbush, UK) and haematocrit (Hawksley, Lancing, UK) concentrations for the determination of plasma volume change (Dill and Costill, 1974). The remaining EDTA-treated blood was then spun for centrifugation under 2000 xg for 10 min at 4°C (Heraeus Primo R; Thermo Fisher Scientific, Loughborough, UK) for plasma extraction then stored at -80°C for later analysis of plasma albumin concentration using an automated spectrophotometric analyser (RX Daytona, Randox, Crumlin, Ireland). Plasma albumin content was consequently determined from plasma albumin concentration and the percentage change in plasma volume. Participants were assumed to have a plasma volume (in litres) equivalent to 5 % of body mass (in kg) at baseline and plasma volume at follow-up was determined using baseline plasma volume and the relative change in plasma volume, as previously reported (James *et al.*, 2014).

### **Muscle biopsy sampling**

A needle biopsy technique (Bergstrom, 1962) under local anaesthetic (1 % lidocaine; Hameln Pharmaceuticals Ltd., Brockworth, UK) was used to obtain muscle tissue. A 2-3 mm skin incision was made from the lateral portion (*vastus lateralis*) of the thigh from the same leg at baseline and follow-up and separated by 2-3 cm, with the use of dominant/non-dominant legs being counterbalanced between participants.

**Muscle tissue sample processing**

Once removed from the thigh, each muscle sample was immediately immersed in liquid nitrogen and kept stored -80°C pending analysis. Subsequently, samples were defrosted and transferred into an RNase-free conical tube (Corning, Ewloe, UK) containing 2 ml of Trizol (Invitrogen, Paisley, UK) and spun at 4000 g for 60 seconds at 4°C before 400 µl of chloroform was added to the mixture. After shaking the mixture vigorously for 15 seconds samples were incubated at room temperature for 3 min and then centrifuged at 4000 g for 15 min at 4°C. The aqueous phase was removed to a fresh conical tube and used for gene expression. The aqueous phase was mixed with 1500 µl of 100 % ethanol before being loaded in an RNeasy mini column (Qiagen, Manchester, UK). Thereafter, the RNA was eluted using 25 µl of RNase-free water and 2 µl were then utilised for RNA quantitation using spectrophotometry (Spectrostar Nano, BMG Labtech, Ortenberg, Germany), with 400 ng of total RNA reverse transcribed using cDNA reverse transcription kit (Superscript III, Invitrogen, Paisley, UK).

**Quantitative real-time PCR**

Taqman low-density custom array using Micro Fluidic cards (Applied Biosystems, Warrington, UK) was used for the relative quantification of expression of selected metabolic genes. Each card allowed for eight samples to be run in parallel against Taqman gene expression assay targets that were pre-loaded into each of the wells on the card. Briefly, 50 µl of Taqman Universal PCR master mix (Applied Biosystems, Warrington, UK) was added to 200 ng of cDNA into an Eppendorf RNase-free tube. RNase-free water was added to make the total volume of the reaction mixture up to 100 µl. The reaction mixture was mixed, centrifuged and loaded into one of the fill reservoir of the Micro Fluidic card. The cards were centrifuged (MULTIFUGE 3 SR; Heraeus, Thermo Fisher Scientific, Loughborough, UK) and ran on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Relative quantification of the genes of interest was performed using the comparative CT method. The average expression of two housekeeping genes [actin, alpha 1 (ACTA1) and hydroxymethylbilanesynthase (HMBS)] and baseline were used to normalise the data.

### Standardisation of lifestyle

Throughout the duration of the study, participants were asked to maintain their habitual dietary energy intake and activity levels (i.e. no substantial changes in caloric intake, dietary habits or physical activity levels outside the scope of the prescribed supplements and training sessions). Over the 48 h prior to baseline testing, a weighed dietary record and an exercise activity log was completed by participants. These dietary records were analysed using nutritional analysis software (Nutritics LTD, Dublin, Ireland) and were not different between the CHO (mean  $\pm$  SD;  $10092 \pm 2117 \text{ kJ}\cdot\text{d}^{-1}$ ;  $51 \pm 7 \%$  carbohydrate;  $30 \pm 8 \%$  fat;  $19 \pm 5 \%$  protein) and CHO-P ( $9699 \pm 2448 \text{ kJ}\cdot\text{d}^{-1}$ ;  $51 \pm 7 \%$  carbohydrate;  $32 \pm 7 \%$  fat;  $17 \pm 2 \%$  protein) groups. Participants also abstained from alcohol consumption and refrained from strenuous physical activity, with any light exercise recorded and matched during the period of standardisation (48 h) of lifestyle for follow-up testing. Moreover, three x 3-day dietary records were assessed and subsequently analysed from participants at different intervals during the six-week training intervention period. This was aimed to provide a reflection of dietary intake habits between groups during the training period.

During prescribed training days, participants were required not to perform any other structured exercise session and were instructed to ingest a meal (breakfast or lunch depending on the time of day) approximately 2 h prior to any session and thereafter abstain from any caloric intake prior to the commencement of the training session. Participants in both groups were also instructed not to consume any energy outside the prescribed supplements during the designated supplement provision periods (2 h immediately post-exercise). A checklist was included to confirm supplement intake, time of ingestion and avoidance of any caloric intake outside the scope of the prescribed supplements. This was aimed to ensure adherence to the nutritional intervention intake /timing throughout the training period.

To accurately monitor training and ultimately standardise the training load between treatments *in situ*, an exercise diary was provided to each participant. Participants completed the relevant information relating to the exercise session (time of day the training session was started, total duration of session) on any of the training days, which was then be verified by an electronic monitoring system that requires participants to use a key card to enter and leave

the gymnasium. The exercise logs were collected and cross-examined with the electronic monitoring system to verify adherence for each exercise session attendance/date/duration.

### Statistical analysis

A *priori* sample size estimation (G\*power version 3.1.7, University Düsseldorf, Düsseldorf, Germany) was performed using data similar to the current study work (Ferguson-Stegall *et al.*, 2011a) and revealed that a total of 24 participants were required to achieve 90 % power to detect a worthwhile increase in endurance capacity (i.e.  $\dot{V}O_{2\max}$ ) of  $5.3 \text{ ml}\cdot\text{kg}^{-1}$  and standard deviation of  $3.3 \text{ ml}\cdot\text{kg}^{-1}$  using a two-tailed *t*-test for independent means with an alpha level of 0.05. Rolling recruitment of  $\approx 30$  participants was therefore conducted in consideration of an anticipated 15 % drop-out rate (Ferguson-Stegall *et al.*, 2011a; Liu *et al.*, 2012). The majority of data include measurements of markers of endurance training adaptations at baseline and follow-up, and thus interactions were explored using a two-way linear mixed model with repeated measures (time  $\times$  trial) to identify overall differences between experimental conditions with time (pre- and post-training) and trial (CHO-P and CHO) inserted as covariates. Any data that require a single comparison of two means were tested for normality using the Shapiro-Wilk test before using an independent *t*-test or non-parametric equivalent to examine differences between treatment arms (e.g. magnitude of change in  $\dot{V}O_{2\max}$  between treatments). Relationships between outcome variables was examined using a Pearson's product-moment correlation and any meaningful associations (i.e.  $r \geq 0.7$ ) were further explored by post-stratification of the treatment group according to baseline status. Significance was set at  $p \leq 0.05$  and all results are reported as the mean  $\pm$  standard deviation (SD) unless stated otherwise.

## Results

### Participant characteristics

There were no baseline differences ( $p > 0.05$ ) between CHO and CHO-P groups for any variable reported in Table 7.2. Thirty-three participants were initially recruited to take part in the study (Figure 7.1). However, three participants did not meet the eligibility criteria and

were excluded from the outset. Furthermore, five participants withdrew from the study, two of whom sustained musculoskeletal injuries unrelated to the training intervention. The other two individuals reported time commitments as a reason for withdrawing from the study, whilst a single individual did not provide any information for their withdrawal; thus 25 participants completed the study. Mean adherence to the exercise intervention (using the exercise logs and gym-based electronic system) was 96 % (SD 4 %). Consequently, from the 26 prescribed exercise sessions, participants completed  $25 \pm 1$  and  $25 \pm 1$  in both CHO-P and CHO groups ( $p = 0.71$ ).

Table 7.2 shows the characteristics of the chosen cohort of participants before and after training and no time  $\times$  trial interactions were identified in any variable between groups ( $p > 0.05$ ). Moreover, no changes from baseline to post-intervention in anthropometric measures were shown in either group. Resting and maximal heart rates were reduced following the intervention in both treatments ( $p < 0.01$ ), with no difference in these responses between groups. Assessments of baseline resting metabolic rate were closely matched between the CHO-P and CHO groups (Table 7.2) and were stable within  $\approx 155 \text{ kJ}\cdot\text{day}^{-1}$  from baseline to follow-up. Furthermore, no notable differences were shown between groups at follow-up ( $243 \text{ kJ}\cdot\text{day}^{-1}$ ;  $p = 0.6$ ). Similarly, the hydration status of participants via urine osmolality measurements was similar across groups, with no differences between groups at follow-up.

**Table 7.2** Participant characteristics pre and post-training.

	<u>CHO-P</u>		<u>CHO</u>	
	Pre	Post	Pre	Post
Age (y)	20 ± 2	–	20 ± 2	–
Height (cm)	179 ± 10	–	179 ± 10	–
Body mass (kg)	77.7 ± 13	76.0 ± 11	73.9 ± 9	74.0 ± 8
Body fat (%)	15.8 ± 5.2	15.4 ± 3.9	17.0 ± 3.8	16.4 ± 3.4
BMI (kg·m <sup>2</sup> )	24.3 ± 3.4	23.8 ± 2.7	23.1 ± 2.0	23.1 ± 1.7
Resting heart rate (beats·min <sup>-1</sup> )	63 ± 6	57 ± 8 <sup>*</sup>	59 ± 7	54 ± 10 <sup>*</sup>
Maximum heart rate (beats·min <sup>-1</sup> )	200 ± 12	195 ± 10 <sup>*</sup>	206 ± 7	199 ± 7 <sup>*</sup>
$\dot{V}O_{2\max}$ (L·min <sup>-1</sup> )	4.16 ± 0.5	4.38 ± 0.6 <sup>*</sup>	4.06 ± 0.5	4.29 ± 0.5 <sup>*</sup>
$\dot{V}O_{2\max}$ (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	54.5 ± 6	57.7 ± 5 <sup>*</sup>	55.1 ± 5	58.0 ± 4 <sup>*</sup>
Resting metabolic rate (kJ·day <sup>-1</sup> )	7686 ± 322	7849 ± 238	7757 ± 301	7607 ± 301
Urine osmolality (mOsm·kg <sup>-1</sup> )	523 ± 349	584 ± 291	516 ± 359	600 ± 282

Values are means ± SD. \*= Significant effect of time after six weeks of training ( $p < 0.01$ ).

### Maximal oxygen uptake

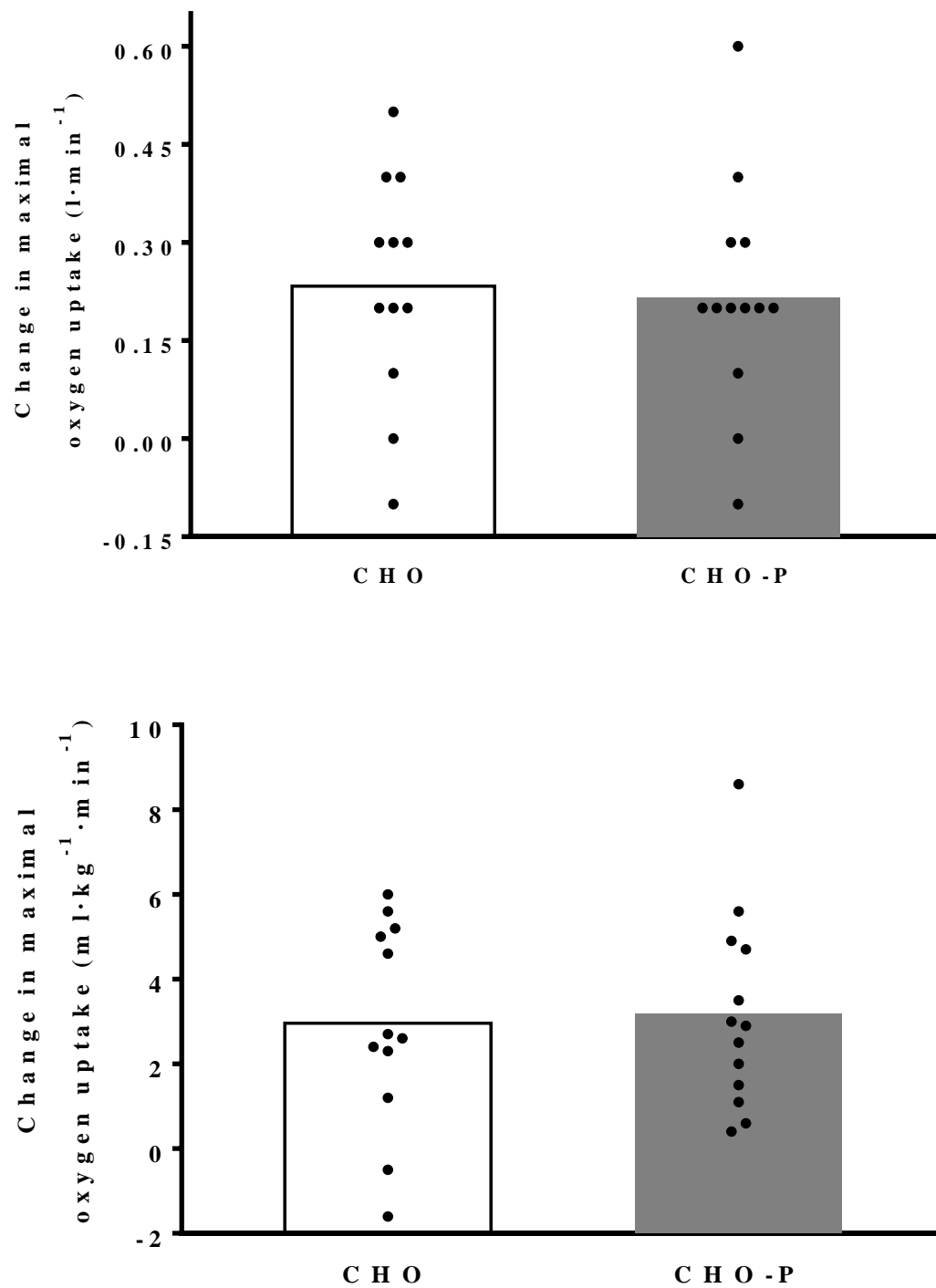
Absolute and relative  $\dot{V}O_{2\max}$  of the study population significantly improved in response to six weeks of treadmill running by  $0.2 \text{ L}\cdot\text{min}^{-1}$  and  $3.0 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  ( $p < 0.001$ ). The magnitude of improvement from baseline to follow-up in both absolute and relative  $\dot{V}O_{2\max}$  was not different between groups (Table 7.2), with improvements from baseline of  $5.3 \pm 4 \%$  and  $5.3 \pm 4 \%$  in CHO-P and CHO treatments, respectively. Consequently, the change in both absolute and relative  $\dot{V}O_{2\max}$  was not different between groups, as reflected by no time  $\times$  trial interactions ( $p > 0.05$ ; Figure 7.3).

### Plasma volume and blood constituents

Table 7.3 presents plasma volume estimations and blood parameters between groups. A significant time  $\times$  trial interaction was observed in plasma albumin content ( $F = 6.96$ ;  $p = 0.01$ ). Thus, there was an increase of plasma albumin content of  $0.18 \text{ g}\cdot\text{kg}^{-1}$  following the intervention in the CHO-P group ( $p = 0.01$ ), whilst no change was identified in CHO group in response to the intervention.

There was no between-group effect on plasma volume normalised to include an estimation of baseline plasma volume (i.e. 5 % of total body mass). However, as shown in Table 7.3, a time effect was shown whereby an increase in plasma volume by 0.2 L in CHO-P treatment ( $p = 0.03$ ) when compared to 0.06 L in the CHO treatment following the intervention. Although there was a trend for a higher percent change in plasma volume in CHO-P group ( $7 \% \pm 6 \%$  *versus*  $3 \% \pm 3 \%$  in CHO group) this was not statistically significant ( $p = 0.07$ ). The change in plasma volume following training was significantly correlated with changes in plasma albumin content ( $r = 0.77$ ;  $p < 0.001$ ).





**Figure 7.3** Absolute and relative change in maximal oxygen uptake at baseline and follow-up in CHO and CHO-P groups. Values are mean and individual scores for each participant.

**Table 7.3** Plasma volume and blood constituents before and after six weeks of training.

	<u><b>CHO-P</b></u>		<u><b>CHO</b></u>	
	<b>Pre</b>	<b>Post</b>	<b>Pre</b>	<b>Post</b>
<b>Haemoglobin (g·dl<sup>-1</sup>)</b>	14.8 ± 1.0	14.4 ± 0.80	14.5 ± 0.70	14.6 ± 0.90
<b>Haematocrit (%)</b>	42.8 ± 2.3	41.4 ± 1.8	42.7 ± 2.3	41.8 ± 3.1
<b>Plasma albumin concentration (g·dl<sup>-1</sup>)</b>	41.8 ± 1.8	42.3 ± 1.7	41.7 ± 1.7	41.2 ± 2.2
<b>Plasma albumin content (g·kg<sup>-1</sup>)</b>	2.09 ± 0.09	2.27 ± 0.20 <sup>*†</sup>	2.08 ± 0.09	2.08 ± 0.13
<b>Plasma volume (L)</b>	3.88 ± 0.70	4.10 ± 0.80 <sup>*</sup>	3.68 ± 0.40	3.74 ± 0.60

Values are mean ± SD. \* = Significant effect of time after six weeks of training ( $p < 0.05$ ). † = Significant difference between treatments at follow-up ( $p = 0.01$ ).

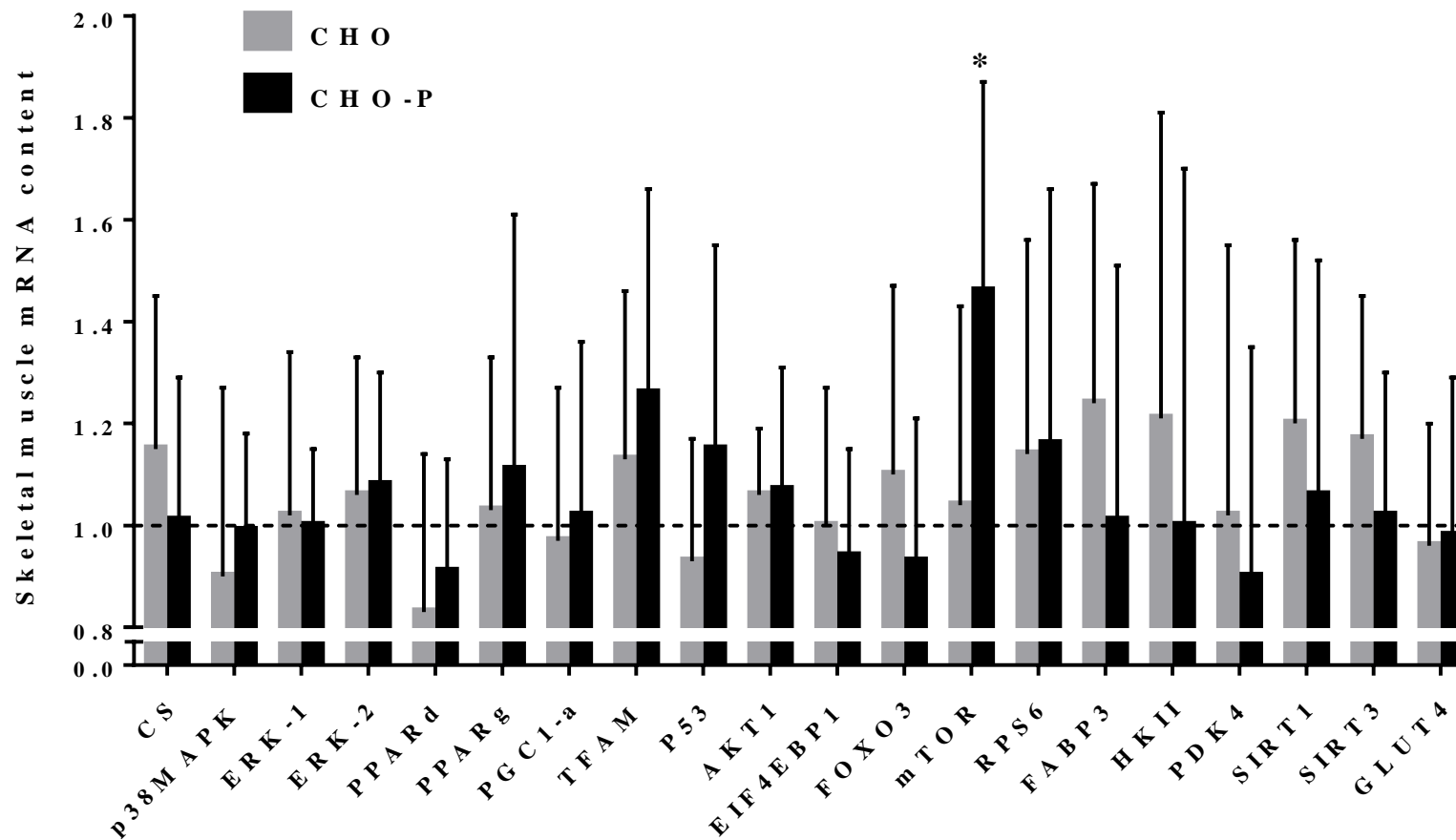
### Intramuscular parameters

Figure 7.3 illustrates the change in expression of key genes related to endurance training adaptation in CHO ( $n=7$ ) and CHO-P group ( $n=8$ ). An up-regulation in the expression of mTOR was shown from pre- to post-intervention in only the CHO-P group ( $p<0.05$ ). No differences in the change in expression of other measured metabolic genes were observed between the CHO and CHO-P treatments ( $p>0.05$ ).

### Diet analysis

Dietary intake estimated by three x 3-day dietary records during the training intervention did not reveal any differences between groups ( $p=0.62$ ). Therefore, total energy intake and percentage contribution of macronutrients were similar between the CHO (mean  $\pm$  SD;  $9920 \pm 2979$  kJ $\cdot$ d $^{-1}$ ;  $51 \pm 6$  % carbohydrate;  $29 \pm 5$  % fat;  $17 \pm 2$  % protein) and CHO-P ( $9301 \pm 2494$  kJ $\cdot$ d $^{-1}$ ;  $47 \pm 6$  % carbohydrate;  $30 \pm 4$  % fat;  $20 \pm 5$  % protein) groups excluding the intake of nutritional supplements, which were identical in energy content (Table 7.1). Overall, the dietary intake excluding supplementation was not different during the training intervention and the 48 h baseline dietary records ( $p>0.05$ ).

Total energy intake with supplementation was therefore  $11991 \pm 2916$  kJ $\cdot$ d $^{-1}$ ;  $62 \pm 5$  % carbohydrate;  $24 \pm 4$  % fat;  $14 \pm 1$  % protein) in CHO group, and  $11380 \pm 2527$  kJ $\cdot$ d $^{-1}$ ;  $50 \pm 5$  % carbohydrate;  $25 \pm 4$  % fat;  $25 \pm 5$  % protein) in CHO-P group. The macronutrient intake relative to body mass was  $6.1 \pm 1.3$  g carbohydrate $\cdot$ kg BM $^{-1}\cdot$ d $^{-1}$ ;  $1.1 \pm 0.4$  g fat $\cdot$ kg BM $^{-1}\cdot$ d $^{-1}$ ;  $1.4 \pm 0.3$ g protein $\cdot$ kg BM $^{-1}\cdot$ d $^{-1}$  in the CHO group and ( $4.6 \pm 1.1$  g carbohydrate $\cdot$ kg BM $^{-1}\cdot$ d $^{-1}$ ;  $1.1 \pm 0.4$  g fat $\cdot$ kg BM $^{-1}\cdot$ d $^{-1}$ ;  $2.3 \pm 0.5$  g protein $\cdot$ kg BM $^{-1}\cdot$ d $^{-1}$  in CHO-P group. Thus, relative daily carbohydrate intake was higher in CHO group ( $p=0.016$ ) and the amount of protein ingested was higher in the CHO-P group ( $p<0.001$ ).



**Figure 7.4** Relative gene expression represented as fold change from baseline for several key genes related to mitochondrial biogenesis, muscle protein synthesis and carbohydrate/lipid metabolism. Values are mean  $\pm$  SD. \*,  $p < 0.05$  baseline *versus* follow-up

## Discussion

The current study is the first to examine the effect of post-exercise carbohydrate-protein supplementation *versus* energy matched carbohydrate ingestion upon the adaptive response to long term (i.e. six weeks) running-based endurance training. While  $\dot{V}O_{2\max}$  increased following six weeks of treadmill running, there were no differences in the magnitude of improvement in  $\dot{V}O_{2\max}$  between the two treatment arms. However, post-exercise carbohydrate-protein ingestion increased plasma albumin content following training relative to an energy matched post-exercise carbohydrate solution.

In accordance with the above, it is reasonable to suggest that  $\dot{V}O_{2\max}$  trainability is a single, inherently heterogenic indicator of endurance training adaptations and other physiological parameters should be considered in conjunction with this variable to assess overall endurance training adaptation. Indeed,  $\dot{V}O_{2\max}$  trainability may encompass a considerable genetic component, but differences in initial  $\dot{V}O_{2\max}$ , age and testing modality (i.e. running *versus* cycling) have also been implicated to influence  $\dot{V}O_{2\max}$  (Basset and Boulay, 2000; Bouchard *et al.*, 1999; Bouchard and Rankinen, 2001). These factors may provide some explanation for the discrepant findings between the current investigation and those reporting an increase in the magnitude of improvement in  $\dot{V}O_{2\max}$  with post-exercise protein ingestion (Ferguson-Stegall *et al.*, 2011a; Robinson *et al.*, 2011). Thus, in contrast to the current study, participants were older (Robinson *et al.*, 2011), had noticeably lower pre-training  $\dot{V}O_{2\max}$  scores and used cycle ergometry as an exercise modality to measure  $\dot{V}O_{2\max}$  (Ferguson-Stegall *et al.*, 2011a; Robinson *et al.*, 2011). Nevertheless, others did not demonstrate further improvement in  $\dot{V}O_{2\max}$  with post-exercise protein feeding (Cramer *et al.*, 2012; Okazaki *et al.*, 2009b), despite using a similar cohort of participants and exercise modality to those reporting further improvements in  $\dot{V}O_{2\max}$  when endurance training is coupled with post-exercise protein ingestion (Ferguson-Stegall *et al.*, 2011a; Robinson *et al.*, 2011). It should be recognised, however, that the experimental design of these studies (Cramer *et al.*, 2012; Okazaki *et al.*, 2009b) limits our understanding on the precise role of post-exercise protein ingestion through the addition of other nutrients in the post-exercise supplement and the

absence of an energy-matched macronutrient treatment arm. Notwithstanding these limitations, the present findings concur with the latter view that protein feeding does not potentiate improvements in  $\dot{V}O_{2\max}$  with six weeks of endurance training in young healthy adults.

Plasma volume expansion is known to occur following submaximal endurance exercise training (Goodman *et al.*, 2005; Green *et al.*, 1991; Mier *et al.*, 1996), which is mainly ascribed to an increase in plasma albumin content and thus retention of fluid within the intravascular space (Convertino *et al.*, 1980). Moreover, a significant correlation between the change in training-induced plasma volume and albumin content has been reported ( $r^2 = 0.69$ ;  $p < 0.05$ ) consistent with the possibility that chronic hypervolemia may be oncotically mediated (Okazaki *et al.*, 2009b). The latter would explain the present findings as changes in plasma volume following training were significantly correlated with changes in plasma albumin content ( $r = 0.77$ ;  $p < 0.001$ ). However, differences may exist between the employed method of calculating plasma volume change and other techniques that measure absolute plasma volume such as Evans blue dye dilution (Gillen *et al.*, 1991). Nonetheless, calculations of plasma volume that are based on haematocrit change correlate well ( $r = 0.99$ ) with data acquired from direct measurement of plasma volume (Greenleaf *et al.*, 1979). Indeed, deviations in plasma osmolality from normal values can affect the measurement of haematocrit (Watson and Maughan, 2014), albeit these generally occur under circumstances that were not present in the current study such as intense exercise, severe dehydration or ingestion of large volumes of diluted beverages (Maughan and Leiper, 1995). In this study all participants consumed 500 ml water prior to arrival to the laboratory and were fully rested for 15 min prior to the venous blood sample. Additionally, the coefficient of variance (CV %) for haemoglobin and haematocrit measurements were  $< 0.9\%$ , which is below day-to-day variability ( $\approx 1.3\%$ ) in plasma volume (Greenleaf *et al.*, 1979). It is therefore reasonable to suggest that the calculated plasma volume expansion from baseline to follow-up in the current study can be ascribed to endurance training and post-exercise feeding intervention.

An increase in plasma albumin content was only shown with the carbohydrate-protein supplement in the current experiment and thus the mechanism behind this increase with post-exercise protein supplementation warrants further discussion. It has been consistently

demonstrated that hepatically derived plasma protein synthesis is stimulated by the acute ingestion or infusion of amino acids (Caso *et al.*, 2007; De Feo *et al.*, 1992; Moore *et al.*, 2009). Moreover, this acute response can translate into a chronic change in plasma albumin content when endurance training is coupled with post-exercise carbohydrate-protein ingestion (Okazaki *et al.*, 2009b). The current data support the latter mechanism by demonstrating an increase in plasma albumin content when protein was co-ingested following exercise training. In conjunction with amino acid availability, insulin was shown to affect hepatic protein synthesis (Ahlman *et al.*, 2001; De Feo *et al.*, 1991) and an insulin stimulation of  $\approx 150$  pmol/L was thought to maximise hepatic albumin synthetic response (Caso *et al.*, 2007). Indeed, the known synergetic effect of carbohydrate and protein co-ingestion on insulin secretion is well documented (van Loon *et al.*, 2000a). Coupled with the provision of protein in amounts ( $0.4 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) sufficient to maximise albumin synthetic response (Moore *et al.*, 2009), the increase in plasma albumin content is likely to be attributable to the inclusion of protein following exercise in the present study. In support of this, it has been suggested that  $\approx 1 \text{ g}$  of albumin binds to 18 ml of water in plasma (Scatchard *et al.*, 1944) and therefore the 218 ml expansion of plasma volume in CHO-P trial in the current study could be entirely attributable to the increase in plasma albumin content ( $0.18 \text{ g} \cdot \text{kg}$ ). Nevertheless, this may partially explain the observed expansion in plasma volume and concomitant increase in plasma albumin in the absence of post-exercise insulin and hepatic fractional synthetic rate measurements.

Another plausible mechanism for the increase in plasma albumin content is the absolute increase in protein intake (per day) in CHO-P trial relative to CHO. Indeed, it was reported that reducing daily protein intake from 1 to 0.75 and  $0.5 \text{ g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  reduces the fractional synthetic rate of albumin in a dose-dependent manner (Thalacker-Mercer *et al.*, 2007). These findings may suggest that the increase in plasma albumin content in the current study may be related to the absolute daily intake of protein ( $2.3 \text{ g protein} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  *versus*  $1.4 \text{ g protein} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  in CHO-P and CHO, respectively) irrespective of the timing of the post-exercise supplementation. However, albumin content was shown to increase with post-exercise carbohydrate-protein ingestion during the first 2 h of recovery but remained at this level during the remainder of 23 h despite the ingestion of meals containing higher absolute amounts of protein than the post-exercise nutritional supplement (Okazaki *et al.*, 2009a). Collectively, these findings suggest that the timing of protein ingestion following exercise

and total daily protein intake can be an important factor in stimulating albumin synthesis and may lend support to the present findings of increased plasma albumin content with the inclusion of protein in a post-exercise carbohydrate supplement.

A second important factor in determining  $\dot{V}O_{2\max}$  is the capacity of the contracting muscle to extract and utilise the oxygen from circulation allowing greater capacity to generate ATP through oxidative phosphorylation (Rivera-Brown and Frontera, 2012), which is driven predominantly by an increase in mitochondrial biogenesis (Hood *et al.*, 2006). In the current investigation, no differences were detected in the expression of genes related to mitochondrial biogenesis or enzyme activity related to oxidative phosphorylation. However, the muscle samples during the study were taken following 48 h of lifestyle standardisation and thus any transient peaks in endurance exercise specific genes may have occurred, given that up-regulation of these genes peaks at 10-24 h following an acute exercise bout (Leick *et al.*, 2010). Indeed, it was previously reported that two weeks of post-exercise protein supplementation may enhance peroxisome proliferator-activated receptor gamma co-activator-1 $\alpha$  (PGC-1 $\alpha$ ) mRNA expression measured 6 h following an acute exercise bout (Hill *et al.*, 2013). Due to the experimental design of the latter study, however, it is impossible to distinguish whether these effects were related to chronic or acute supplementation given that these measurements followed acute post-exercise ingestion with varied macronutrient intakes (carbohydrate *versus* carbohydrate-protein). Nonetheless, transient, pulsed increases in relevant gene expression lead to increases in transcriptional and mitochondrial proteins (Perry *et al.*, 2010). In support of this, an increase in the protein content of markers of mitochondrial biogenesis (PGC-1 $\alpha$ ) have been reported following 4.5 weeks of endurance exercise training. However, the magnitude of improvement was similar irrespective of the addition of protein in a post-exercise carbohydrate supplement (Ferguson-Stegall *et al.*, 2011a). While the protein content of the markers of mitochondrial biogenesis were not assessed in the present investigation, the available evidence indicates that any nutrient-specific enhancements in  $\dot{V}O_{2\max}$  following endurance training are not associated with further increase in markers of mitochondrial biogenesis.

There are a number of limitations to the present study. A non-exercising control group or a group that commenced the training with no post-exercise supplementation (i.e. placebo group)



was not included in the current study. Thus, it is not possible to directly assess the effect of training stimulus/nutrient intake *per se* on the outcome measures obtained. Furthermore, full dietary control throughout the training period was not employed. Although 3 x 3-day diet records were obtained during the six week training period, it is entirely possible that within and between-group variations in macro and micronutrient intakes on non-recorded days may have influenced the adaptive response. Moreover, it is not possible to objectively determine participants' physical activity outside the scope of the training intervention (e.g. days where prescribed training was not undertaken), which may also have an influence in the obtained measurements. Finally, the current experiment explored relative change in the expression of selected genes associated with endurance training adaptation, and thus the findings related to intra-muscular adaptive response to endurance training plus post-exercise supplementation are only limited within the context of this measure. Therefore, it remains feasible that the magnitude of increase in transcriptional and mitochondrial proteins may have been different between the experimental groups.

In conclusion, post-exercise carbohydrate-protein ingestion during six weeks of treadmill run training resulted in an increase in plasma albumin content. While the training intervention improved  $\dot{V}O_{2\max}$ , the magnitude of this effect was not different with the inclusion of protein in a post-exercise recovery solution when compared to an isocaloric carbohydrate supplement.

## CHAPTER 8

### GENERAL DISCUSSION

#### 8.1 Introduction

Optimising short-term recovery is an important consideration for both athletes who train and compete with limited time to recover and recreational exercisers to help them avoid any residual fatigue which could negatively influence their capacity to partake in exercise. Moreover, post-exercise recovery from the stress of exercise is recognised as an essential component of any periodised training programme aimed at maximising training-induced adaptation and consequently overall exercise performance/capacity. The research presented in this thesis has built on existing knowledge by examining muscle glycogen metabolism during short-term recovery and a repeated exhaustive exercise bout with different nutrient intakes/compositions. In addition, the current work explored whether co-ingesting protein with carbohydrate following exercise would translate into an amplified adaptive response to running-based endurance exercise training. This chapter will collate and consider the findings from the experimental chapters in this thesis.

#### 8.2 Muscle glycogen availability and the capacity for repeated exercise

Pre-exercise muscle glycogen availability is known to influence the capacity for exercise during an initial exercise bout. However, whether muscle glycogen mediates such an effect on repeated exercise capacity following limited recovery remained unknown. The experimental procedures in **Chapter 4** indicate that increasing carbohydrate intake from 0.3 to 1.2 g·kg BM<sup>-1</sup>·h<sup>-1</sup> resulted in higher muscle glycogen availability at the end of 4 h recovery, which concurrently improved subsequent endurance capacity in a dose-dependent manner. In this study, comparisons were made late in the repeated exercise bout not only at the time of absolute fatigue between treatment arms, but also the time-point in H-CHO that coincided with fatigue in L-CHO. At this particular time point, muscle glycogen concentrations were higher in H-CHO *versus* L-CHO treatment. The extended run to exhaustion in H-CHO was associated with further utilisation of muscle glycogen and absolute fatigue in both treatments was associated with similar and critically low muscle glycogen concentrations. The findings

therefore suggest that the availability of skeletal muscle glycogen is an important factor in the restoration of endurance capacity because fatigue during repeated exercise was associated with a critically low absolute muscle glycogen concentration. Through the manipulation of carbohydrate availability during short-term recovery to understand the role of muscle glycogen, it can also be concluded that carbohydrate ingestion can be employed to impact repeated exercise capacity through this mechanism.

### **8.3 The addition of protein to carbohydrate and muscle glycogen metabolism during recovery and subsequent exercise**

Protein co-ingestion has been suggested to accelerate muscle glycogen resynthesis relative to carbohydrate only during short-term recovery, but this finding is far from conclusive. Importantly, only two investigations used running as the mode of exercise (Betts *et al.*, 2008; Lunn *et al.*, 2012), and none explored muscle glycogen resynthesis following an exhaustive initial exercise bout that sufficiently deplete muscle glycogen. To add to this, whether carbohydrate-protein ingestion elicits maximal rates of muscle glycogen resynthesis relative to an energy-matched carbohydrate provided in amounts ( $\geq 1 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) sufficient to maximise short-term recovery from running has not been investigated. Beyond muscle glycogen resynthesis, carbohydrate-protein ingestion has been reported to exert an ergogenic benefit upon subsequent exercise, with no plausible physiological mechanism for this effect.

The results from **Chapter 5** report that protein co-ingestion with carbohydrate during short-term recovery equally stimulates muscle glycogen repletion when compared with an isocaloric solution containing carbohydrate in amounts previously reported to maximise muscle glycogen resynthesis (i.e.  $1.2 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ). Despite similar glycogen resynthesis rates, a higher insulin concentration was noted during recovery with the addition of protein to carbohydrate. It can therefore be inferred that when ample amounts of carbohydrate (i.e.  $\approx 1.2 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) are ingested, further elevations in insulin concentrations does not further increase muscle glycogen resynthesis during post-exercise recovery. However, this augmented insulin response may be beneficial in other aspects of recovery such as reducing muscle protein breakdown and thus may improve net protein balance (Biolo *et al.*, 1999; Borsheim *et al.*, 2004). Furthermore, elevations in insulin concentrations may be advantageous in liver glycogen storage (Storer *et al.*, 1981), which is an important consideration for post-

exercise recovery (Casey *et al.*, 2000) that received less attention presumably due to methodological limitations.

In contrast to a number of investigations, the capacity for repeated exercise was not further enhanced with carbohydrate-protein ingestion. It was previously speculated that a reduction in muscle glycogen use may be a mechanism by which the ergogenic benefit of carbohydrate-protein ingestion can be explained. The study reported in **Chapter 5** demonstrates that the addition of protein to a post-exercise carbohydrate supplement does not alter net glycogen degradation during a repeated exhaustive exercise bout when compared to an isocaloric carbohydrate supplement. Rather, fatigue during the repeated exercise bout was associated with low muscle glycogen levels. When considered collectively, muscle glycogen appears to be an important factor in fatigue during a repeated exhaustive exercise bout. It can therefore be concluded that co-ingesting protein with carbohydrate may be beneficial to maximise muscle glycogen repletion under circumstances where the amount of carbohydrate that can be ingested is limited (i.e.  $< 1 \text{ g}\cdot\text{kg BM}^{-1}\cdot\text{h}^{-1}$ ). Moreover, the presence of protein in a post-exercise recovery supplement can mediate other relevant aspects of post-exercise recovery nutrition such as promoting muscle protein synthesis and driving tissue repair and remodelling.

#### **8.4 Reliability of time to exhaustion as a measure of human endurance capacity**

To assess human endurance capacity, **Chapters 4** and **5** adopted an exercise time to exhaustion (TTE) as an outcome measure. Although it has been argued that time-trial (TT) performance measure is more reliable than TTE, these assumptions are generally founded on 2 factors: i) there are no real-world sporting events that require an individual to run until the point of exhaustion; and ii) TTE appears to be less reliable than TT in relation to a valid simulation that resembles actual performance in a given sport (Currell and Jeukendrup, 2008). While applied sports science studies mimic ‘real-world’ sporting events and provide useful tool in assessing performance in a sports context, this naturally constrains them to the rules and regulations of a given sports that may be changed or refined periodically.

If we wish to interpret the value of performance measures in ‘real-world’ events, TTE may arguably be more prevalent and have more important bearings in the broader scale. In terms of prevalence, there are clearly more recreational exercisers than athletes, who may present a case that maintaining their running or cycling endurance capacities to be able to engage with other exercisers at the same intensity and for the duration of the activity (such as running outdoors with friends or be able to maintain the capacity to complete a 90 min football match) is paramount. Furthermore, many athletes undertake exercise scenarios similar to TTE tests. For example, pace is often set by the faster athlete with the majority of athletes attempting to sustain this pace for as long as possible before reducing their pace and consequently falling behind the group/athlete. With regards to importance, endurance capabilities defined as “the ability of a muscle group to sustain external forces for long periods of time” are considered as an integral component in improving the occupational tasks of military personnel (Knapik *et al.*, 2009). This underscores the importance of endurance capacity over performance time in this population whereby completing a march as unit is the goal and not performance at maximal individual capabilities of a set task, which could separate them and pose risks for those personnel. Certainly, scientific investigations that measure constants in nature (i.e. establishing the mechanisms of fatigue) should not be overlooked.

The view that TT is a better representation of human performance is of course a valid one, if the sole reason for a performance measure is to produce an outcome measure that can be directly extrapolated to a real-world event. Nonetheless, it should be appreciated that a large number of scientific investigations involve mechanistic variables of human performance and its limitations, be it physiological, metabolic and neuromuscular among others. Fatigue is a complex phenomenon with several contributing factors, such that fatigue may occur simultaneously in several loci in the human body with the relative underlying mechanisms likely to overlap and interact (Ament and Verkerke, 2009; Knicker *et al.*, 2011). It is imperative, therefore, that to investigate this intricate behaviour, means of inducing volitional fatigue through TTE performance measures are important to be included in a controlled environment to establish mechanistic causes of performance limitations and the possible mediating influences of certain nutritional interventions in delaying the onset of fatigue in humans. Accordingly, given that the aims of the current project are mechanistic in nature provides a foundation for the adopted TTE outcome measure.

With the above rationale for using TTE as a measurement tool to assess endurance capacity in the current thesis, **Chapter 6** investigated the test-retest reliability of TTE from both **Chapters 4** and **5**. Based on Run-1 data (i.e. prior to nutrient provision) between the first and second main trials, both absolute (CV= of 5.4 %) and relative (ICC= 0.88) test-retest reliability were deemed acceptable to detect small but worthwhile effects on endurance capacity. Coupled with the fact that absolute bias between the two TTE runs was 4 % (Bland and Altman plots), it was concluded that the employed TTE protocol can be a reliable tool to assess human endurance capacity.

### 8.5 Post-exercise protein ingestion and endurance training adaptation

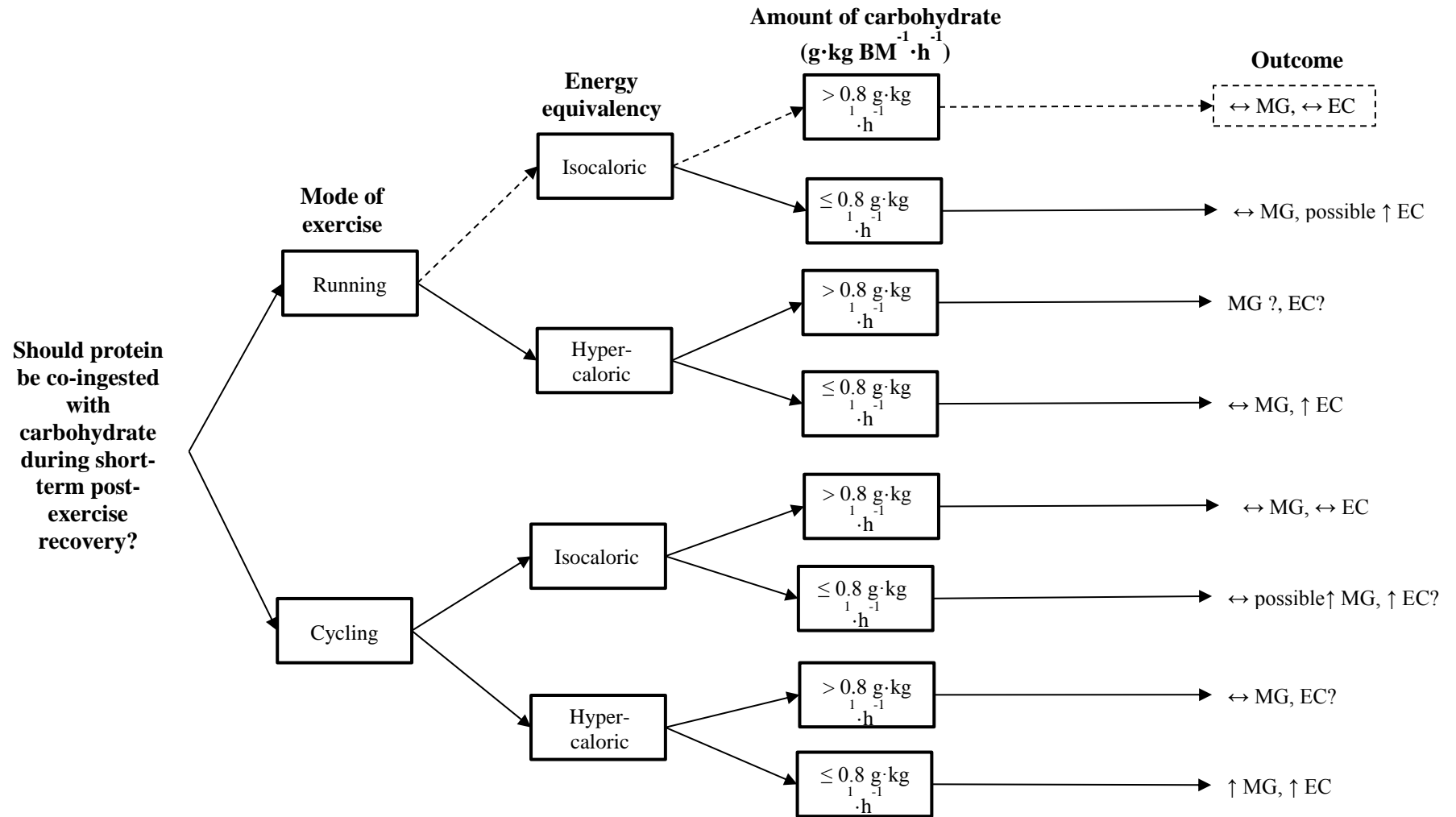
Protein ingestion promotes muscle protein synthesis and thus promotes tissue repair/regeneration and modulate the acute response to training (Ferguson-Stegall *et al.*, 2011b; Hawley *et al.*, 2011; Howarth *et al.*, 2009; Lunn *et al.*, 2012). Based on the aforementioned knowledge, **Chapter 7** was aimed to investigate the influence of post-exercise protein ingestion on the magnitude of adaptation in response to six weeks of endurance training. The data from this study indicate that the addition of protein following each exercise session during training does not translate into an increase in the expression of genes related to endurance training adaptation in the resting fasted state. Furthermore, the increase in  $\dot{V}O_{2\max}$  with training was similar between carbohydrate-protein and an energy-matched carbohydrate control group. Nevertheless, an increase in plasma albumin content at follow-up was only noted in the carbohydrate-protein group, which was associated with an increase in the estimated plasma volume change.

### 8.6 Summary

The present thesis examined the role of carbohydrate and protein ingestion from an initial exhaustive running exercise on muscle glycogen metabolism during short-term recovery and subsequent exercise capacity and influence of long-term post-exercise protein ingestion on the magnitude of adaptation in response to six weeks of endurance training.

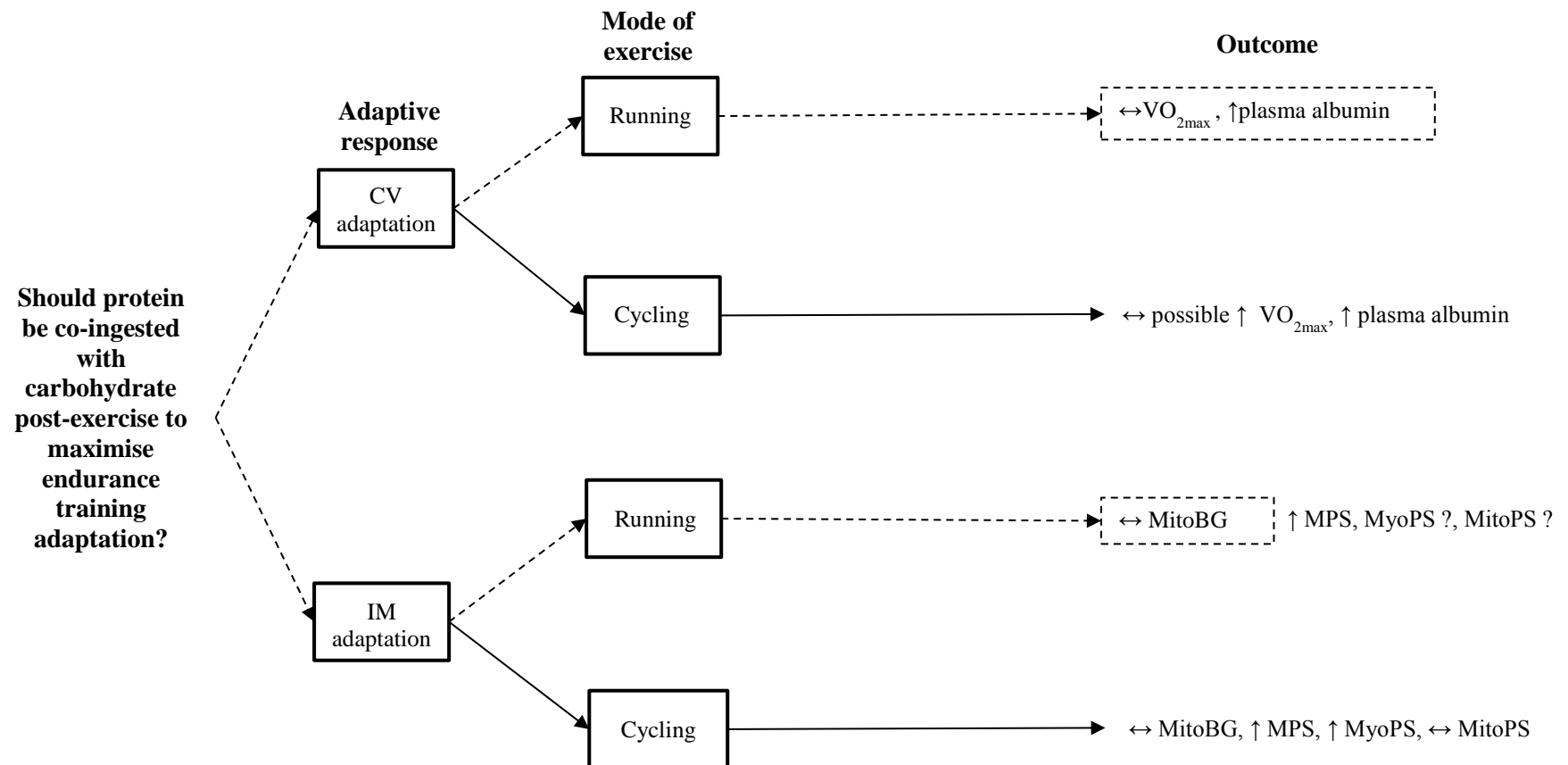
Figures 8.1 and 8.2 illustrate the contribution to knowledge reported in the series of studies in the current thesis in the context of the role of post-exercise carbohydrate and/or protein ingestion on muscle glycogen repletion, the capacity for repeated exercise and its modulation of endurance training adaptation. To summarise collectively, the current work provides the first insight into muscle glycogen metabolism during a repeated exhaustive exercise bout. The findings reported in **Chapter 4** demonstrate that muscle glycogen availability is a major determinant of fatigue during a repeated running exercise bout, and that similar to an initial exercise bout, the onset of fatigue occurred at the point where glycogen levels reached critically low concentrations. Moreover, considerable disparity exists with regards to the co-ingestion of protein to carbohydrate to maximise muscle glycogen repletion and no information is available in regards to its utilisation during a repeated exhaustive exercise bout. The findings in **Chapter 5** provide the first report that the addition of protein to carbohydrate is equally effective in the restoration of muscle glycogen during short-term recovery from exhaustive running when compared to carbohydrate provided in amounts suggested to maximise muscle glycogen resynthesis during short-term recovery. Additionally, it was shown that net muscle glycogen degradation is not influenced by the addition of the protein fraction during recovery, coupled with the absence of any further benefit to restoration of endurance capacity. Rather, the findings from **Chapter 5** corroborate those from **Chapter 4** in that muscle glycogen availability determines the capacity for repeated exercise and thus supports the notion that energy content and not macronutrient composition (at least in the context of protein co-ingestion) determine repeated exercise capacity.

Finally, the current thesis examined the potential for carbohydrate-protein ingestion to optimise cardiovascular adaptations in response to running-based endurance exercise training. **Chapter 7** reports a previously unexplored aspect of nutrient-exercise interactions by examining the role of post-exercise protein ingestion upon the adaptive response to six weeks of treadmill endurance training in young individuals. While the magnitude of improvement in  $\dot{V}O_{2\max}$  was similar when post-exercise carbohydrate-protein was ingested relative to an isocaloric carbohydrate supplement, an increase in plasma albumin content was noted in the former without any further enhancement in the magnitude of endurance training adaptation.



**Figure 8.1** Flow diagram illustrating the influence of post-exercise protein ingestion during short-term recovery. Dashed lines represent the findings from the current thesis (Chapter 5). MG; muscle glycogen resynthesis, EC; endurance capacity.



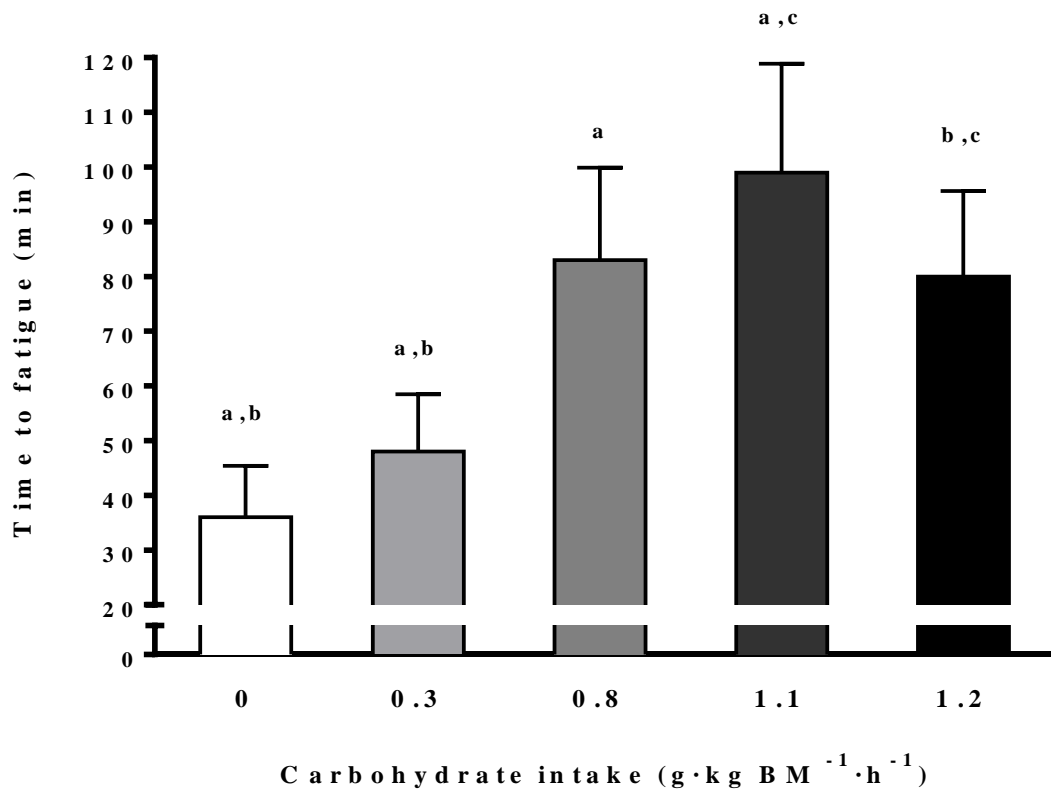


**Figure 8.2** Flow diagram illustrating the influence of post-exercise protein ingestion on endurance training adaptation. Dashed lines represent the findings from the current thesis (Chapter 7). CV; cardiovascular, IM; intramuscular, MPS; mixed muscle protein synthesis, MyoPs; myofibrillar protein synthesis, MitoPS; mitochondrial protein synthesis, MitoBG; mitochondrial biogenesis.

### 8.7 Future directions

In **Chapter 4**, muscle glycogen availability was implicated as a major determinant of fatigue during a reported exercise bout. In this regard, determining the role of liver glycogen availability prior to a repeated exhaustive running bout would be an central to further understanding fatigue mechanisms during repeated exercise, particularly given the importance of liver glycogen in restoration of endurance capacity in cycling (Casey *et al.* 2000). Interestingly, repeated exercise capacity was enhanced by 12 min in L-CHO relative to the familiarisation trial ( $p < 0.01$ ) during Run-2, despite that time to exhaustion was similar at Run-1 between these trials. This certainly argues the possibility that liver glycogen availability may be an important factor in relation to the onset of fatigue during a repeated exhaustive running bout, which is possible to quantify using NMR spectroscopy.

The rate of carbohydrate intake during recovery is an important determinant of muscle glycogen resynthesis. Surprisingly, however, few investigations directly assessed the relative dose-response of carbohydrate ingestion (and indeed feeding frequency) on restoration of muscle glycogen. Rather, the general recommendations on carbohydrate ingestion during short-term recovery are based on comparisons of the rate of glycogen resynthesis relative to the carbohydrate intake across multiple studies. In relation to the latter, exploring varied carbohydrate intakes during limited recovery on restoration of exercise capacity would help further clarify the presence of a dose-response relationship between these two variables (Figure 8.3).



**Figure 8.3** Reported carbohydrate intakes during 4 h recovery and repeated running exercise capacity in Chapter 4 of the thesis (0, 0.3 and 1.2  $\text{g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) and another study (Betts et al. (2007); 0.8 and 1.1  $\text{g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ). Values are mean  $\pm$  SD. Values with similar lower cases are significantly different ( $p < 0.05$ ).

An important finding in **Chapter 5** was that muscle glycogen resynthesis was equally effective in restoring muscle glycogen in the carbohydrate-protein when compared to the energy matched carbohydrate trial (in amounts suggested to elicit maximal muscle glycogen resynthesis rates), despite a greater insulin response in the former. In accordance, a number of interesting questions remain with regards to post-exercise carbohydrate-protein ingestion. This relates to whether the augmented insulin response influences glycogen storage in other insulin-sensitive tissues such as the liver. Another possible avenue of research in this field is to examine whether carbohydrate-protein increases the efficiency of glycogen storage from running relative to an isocaloric carbohydrate when provided in amounts less than those ( $< 1.2 \text{ g} \cdot \text{kg BM}^{-1} \cdot \text{h}^{-1}$ ) suggested to maximise muscle glycogen resynthesis rates. This may be of particular relevance to many recreational exercisers as the aforementioned high carbohydrate intake guidelines are unlikely to be met by this population.

While **Chapter 7** introduces an interesting prospect in the potential role of protein ingestion in optimising cardiovascular training adaptation, a number of follow-up studies are required to further expand on the knowledge obtained from this experiment. For example, it is important to examine whether further increases in  $\dot{V}\text{O}_{2\text{max}}$  would be present with carbohydrate-protein ingestion in a cohort with lower baseline  $\dot{V}\text{O}_{2\text{max}}$  ( $< 50 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) than the current study. Indeed, providing full dietary provision along with undertaking exercise sessions in a controlled laboratory environment throughout the training intervention is future step in confirming the present findings from a free-living experiment. Additionally, introducing treatment arms that do not include any post-exercise supplementation and/or a resting control (no exercise or supplementation) would possibly extend our understanding on the influence of post-exercise nutrient intake/composition of endurance training adaptations. Moreover, the current training study only reported a “snapshot” of the processes involved in intramuscular training adaptations by reporting data pertaining to mRNA targets of mitochondrial biogenesis following 48 h of lifestyle standardisation. Thus, given that the temporal time-course of intramuscular signalling expression in response to nutrition/training is far from understood, it is imperative that

future work explores changes in protein content of genes associated with mitochondrial biogenesis to determine potential nutrient-exercise interactions within skeletal muscle. It is also important to expand investigations regarding acute post-exercise carbohydrate-protein supplementation to other populations such as elderly individuals to broaden our current understanding on the potential role of nutrient-exercise interactions in increasing training efficiency, particularly when considering that post-exercise protein ingestion appears to increase the magnitude endurance training-induced cardiovascular adaptations (Okazaki *et al.*, 2009b; Robinson *et al.*, 2011).

## 8.8 Conclusions

The findings of this thesis indicate that muscle glycogen availability is a major determinant of repeated exercise capacity following short-term recovery, which is consistent with the role of muscle glycogen during an initial prolonged exercise bout. The addition of protein to carbohydrate during short-term recovery equally maximises muscle glycogen repletion relative to an energy-matched carbohydrate, while neither muscle glycogen utilisation nor subsequent exercise capacity are influenced by the addition of the protein fraction. The immediate post-exercise ingestion of carbohydrate-protein increases plasma albumin content in response to six weeks of prescribed running-based endurance training, without any further enhancement in the magnitude of aerobic training adaptations when compared to an energy-matched carbohydrate supplement.

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## **APPENDICES**

### **Appendix A: Pre-trial documentation**

- i. Medical health questionnaire
- ii. Informed consent form
- iii. Profile of mood state

### **Appendix B: Subjective rating scales and profile of mood state**

- i. Rating of perceived exertion scale
- ii. Stomach discomfort scale
- iii. Thirst scale
- iv. Gut fullness scale

### **Appendix C: Lifestyle standardisation**

- i. Food diary booklet
- ii. Exercise activity record
- iii. Training and supplementation checklist (training study)

### **Appendix D: Estimation of protein oxidation rates**

### **Appendix E: Muscle glycogen analysis**

- i. Acid hydrolysis of muscle sample
- ii. Measurement of creatine
- iii. Measurement of ATP, PCr and G-6-P
- iv. Measurement of glucose (insoluble and soluble glycogen)

### **Appendix F: Gene expression analysis**

- i. RNA extraction from muscle tissue
- ii. cDNA conversion from RNA

### **Appendix G: Percent change in plasma volume and plasma albumin content estimation**

### **Appendix H: Statistical procedures**

- i. Calculations of confidence intervals
- ii. Calculation of incremental area under the curve

**Date:**  
**Version 2**

**MEDICAL QUESTIONNAIRE** **Participant**  
**Number** .....

**Project Title:**

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continued well being and (ii) to avoid the possibility of introducing bias into the study outcomes.

Please complete this brief questionnaire to confirm your fitness to participate:

	YES	NO
1. Has your doctor ever said that you have a heart condition or that you should only perform physical activity when recommended by a medical doctor?	<input type="checkbox"/>	<input type="checkbox"/>
2. Do you feel pain in your chest when you perform physical activity?	<input type="checkbox"/>	<input type="checkbox"/>
3. In the past month, have you had chest pain when you were not performing physical activity?	<input type="checkbox"/>	<input type="checkbox"/>
4. Do you lose your balance due to dizziness or do you ever lose consciousness?	<input type="checkbox"/>	<input type="checkbox"/>
5. Do you have a bone or joint problem (e.g. back, knee or hip) that could be made worse by taking part in physical exercise?	<input type="checkbox"/>	<input type="checkbox"/>

	YES	NO
6. Is your doctor currently prescribing drugs for your blood pressure or a heart condition?	<input type="checkbox"/>	<input type="checkbox"/>
7. Do you know of any other reason why you should not take part in physical exercise?	<input type="checkbox"/>	<input type="checkbox"/>
8. Do you currently smoke?	<input type="checkbox"/>	<input type="checkbox"/>
9. Have you ever been diagnosed with a heart arrhythmia?	<input type="checkbox"/>	<input type="checkbox"/>
10. Do you have any dietary allergies (e.g. nuts, milk, etc.)	<input type="checkbox"/>	<input type="checkbox"/>
11. Has your doctor ever said that you have diabetes or any similar metabolic disorder?	<input type="checkbox"/>	<input type="checkbox"/>
12. Do you have any form of bleeding disorder or are taking any medication which can affect the ability of your blood to clot (e.g. Warfarin, aspirin-like drugs, etc.)?	<input type="checkbox"/>	<input type="checkbox"/>
13. Are you aware of having any tendency towards keloid scarring (i.e. scars which expand outside the area of a wound)?	<input type="checkbox"/>	<input type="checkbox"/>
14. Are you currently taking any medication which impacts upon your immune system (e.g. steroids)?	<input type="checkbox"/>	<input type="checkbox"/>
15. Are you aware of any allergies towards stiches?	<input type="checkbox"/>	<input type="checkbox"/>

**Thank You**

## INFORMED CONSENT FORM

Date:

Version 2

**Project Title:****Contact:** Abdullah F. Alghannam

Email: A.F.Alghannam@bath.ac.uk

Tel: 01225 383 448

**Please initial box**

1. I confirm that I have read the participant information sheet dated 17/05/2011 (version 2) relating to the above study and have had the opportunity to ask questions.
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving reason and without my legal rights being affected.
3. I agree to take part in the above study.





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 Name of participant

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 Date

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 Signature

---

 Name of RA/PI

---

 Date

---

 Signature

---

 Name of Witness

---

 Date

---

 Signature

Initials: \_\_\_\_\_

Date: \_\_\_\_\_

**POMS (PROFILE OF MOOD STATES-SHORT FORM)**

Below is a list of words that describe feelings people have. Please read each one carefully. Then

Circle ONE answer to the right, which best describes HOW YOU HAVE BEEN FEELING  
DURING THE PAST 24 HOURS.

The numbers refer to these phrases:

0=not at all

1=a little

2=moderately

3=quite a bit

4=extremely

- |  |  |
|--|--|
| 1. Tense..... 0 1 2 3 4                  | 20. Discouraged..... 0 1 2 3 4           |
| 2. Angry..... 0 1 2 3 4                  | 21. Resentful..... 0 1 2 3 4             |
| 3. Worn out..... 0 1 2 3 4               | 22. Nervous..... 0 1 2 3 4               |
| 4. Unhappy..... 0 1 2 3 4                | 23. Miserable..... 0 1 2 3 4             |
| 5. Lively..... 0 1 2 3 4                 | 24. Cheerful..... 0 1 2 3 4              |
| 6. Confused..... 0 1 2 3 4               | 25. Bitter..... 0 1 2 3 4                |
| 7. Peeved..... 0 1 2 3 4                 | 26. Exhausted..... 0 1 2 3 4             |
| 8. Sad..... 0 1 2 3 4                    | 27. Anxious..... 0 1 2 3 4               |
| 9. Active..... 0 1 2 3 4                 | 28. Helpless..... 0 1 2 3 4              |
| 10. On Edge..... 0 1 2 3 4               | 29. Weary..... 0 1 2 3 4                 |
| 11. Grouchy..... 0 1 2 3 4               | 30. Bewildered..... 0 1 2 3 4            |
| 12. Blue..... 0 1 2 3 4                  | 31. Furious..... 0 1 2 3 4               |
| 13. Energetic..... 0 1 2 3 4             | 32. Full of pep..... 0 1 2 3 4           |
| 14. Hopeless..... 0 1 2 3 4              | 33. Worthless..... 0 1 2 3 4             |
| 15. Uneasy..... 0 1 2 3 4                | 34. Forgetful..... 0 1 2 3 4             |
| 16. Restless..... 0 1 2 3 4              | 35. Vigorous..... 0 1 2 3 4              |
| 17. Unable to Concentrate..... 0 1 2 3 4 | 36. Uncertain about things.... 0 1 2 3 4 |
| 18. Fatigued..... 0 1 2 3 4              | 37. Bushed..... 0 1 2 3 4                |
| 19. Annoyed..... 0 1 2 3 4               |  |

## Rating of Perceived Exertion

6	
7	Very Very Light
8	
9	Very Light
10	
11	Fairly Light
12	
13	Fairly Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Very Very Hard
20	Maximum

## Stomach Discomfort Scale

6	
7	No Discomfort
8	
9	
10	
11	Mild Discomfort
12	
13	
14	
15	Moderate Discomfort
16	
17	
18	
19	Extreme Discomfort
20	



Thirst Scale

6	
7	Not Thirsty
8	
9	
10	
11	Fairly Thirsty
12	
13	
14	
15	Thirsty
16	
17	
18	
19	Very Very Thirsty
20	

Gut Fullness Scale

6	
7	Not Full
8	
9	
10	
11	Fairly Full
12	
13	
14	
15	Full
16	
17	
18	
19	Very Very Full
20	



**Macronutrient Ingestion, Muscle Glycogen & Post-Exercise Recovery**

**Food Diary Information Pack**

**48 hours prior to lab days**

**CONFIDENTIAL**

NAME AND ADDRESS

.....  
.....  
.....  
.....  
.....

AGE.....YRS

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<b>Do</b>	<b>Don't</b>
Weigh & Record your food precisely	Take part if you feel unwell
Adhere to this diet over the 2 days prior to each trial and the day after	Deviate from the specified diet whatsoever
Drink approximately 1 pint of water on the morning of each trial	Have breakfast before any coming into the lab
Arrive in the lab after a 10 h overnight fast	Take part in any strenuous exercise for 48 h prior to a main trial
Bring spare clothes to change into during the recovery period	Consume any alcohol for 48 h prior to any main trial
Bring music to listen to while running	Rush into the lab for any testing

## Food Record Diary

---

- Please record everything you eat and drink (including water) during the 2 days prior to your first visit to the lab. Please try to be as accurate as possible and try to eat your usual diet.
  - For the final meal prior to the lab visits, please also keep to the standard meal that you have been given.
  - We ask that you adhere to the same diet over the 2 days prior to each visit to the lab. Try and think ahead so that you do not eat any foods prior to your first trial which you will not have access to before subsequent trials.
  - Also record if / when you take any food supplements or medication e.g, paracetamol.
  - Please ensure that you **DO NOT** take any sport-related nutritional supplements (e.g. creatine, HMB, etc.)
- 
- If you consume caffeine in your habitual diet, please be aware that the final allowed ingestion of caffeine is **17:00** on the day before any trial.
- 
- Start a new page of the log every day. There is also a notes section at the end of the booklet, this is for you to note any other information you think will be useful e.g. felt unwell.

**One of the most important contributions you can make as a participant in this study is to accurately record your dietary intake and replicate it precisely between trials.**

### INSTRUCTIONS FOR USING THE FOOD DIARY

For solid foods, the food should be placed on the scale on a plate or container. The plate or container must be weighed empty first and the scales can then be zeroed. Each item of food can then be added to the plate and weighed individually, returning the scales to zero between each item.

e.g.	Plate	150g zero scale
	Roast Beef	100g zero scale
	Potato	150g zero scale
	Gravy	30g zero scale

For drinks, a cup or glass must first be weighed and then the scale can be returned to zero and the drink added. Please remember to record separately the weight of tea, milk and sugar put into a drink.

Do not forget to weight and record second helpings and between meal snacks.

Any leftovers (e.g. apple cores) should also be weighed & recorded in the leftovers column.

Eating Out – Most people eat foods away from home each day, please do not forget to record these. Take your diary and scales with you where ever it is possible. If this is too inconvenient just record the type of food eaten with an estimated weight – but please say when a weight has been estimated.

Most snack foods will have the weight of the food on the packet so they do no need weighing if you eat the whole packet yourself.

Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

Start a new page in your diary for each day, and record each item on a separate line. Record the time of day in the first column of each line. Leave a line between different plate entries.

eg. 10.30 am McVities Digestive Biscuits (2) 50g

[illegible]



**Macronutrient Ingestion, Muscle Glycogen & Post-Exercise Recovery**

**Exercise Activity Log**

**48 hours prior to lab days**

**CONFIDENTIAL**

NAME AND ADDRESS

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.....  
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.....

AGE.....YRS

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Do	Don't
Keep your habitual training programme	Perform any vigorous exercise during the 48 h before any trial

- Please record your exercise during the 2 days prior to your first visit to the lab. Please keep to your normal training and avoid any strenuous activity.
- Start a new page of the log every day. There is also a notes section at the end of the log, this is for you to note any other information you think will be useful e.g. felt unwell.

Activity	Begin time	End time	Total minutes



**Post-Exercise Nutrition and Endurance Training Adaptations**

**Exercise and Nutrition Log**

**During Each Training Day**

**CONFIDENTIAL**

NAME AND ADDRESS

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.....  
.....  
.....  
.....

AGE.....YRS

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## Exercise & Nutrition Log

- 
- Please ensure that you fill or tick the relevant boxes during each exercise session.
  - Please ensure that no planned dietary/lifestyle changes occur during the study (e.g. new supplements/diet and/or new exercise regimen).
- 
- Start a new page of the exercise and nutrition log every training week. There is also a notes section on each training week page and at the end of the booklet, this is for you to note any other information you think will be useful e.g. felt unwell.

**One of the most important contributions you can make as a participant in this study is to adhere to your exercise programme in addition to precisely following pre- and post-exercise instructions during each training session.**

**PRE-EXERCISE INSTRUCTIONS:**

Please do not eat within 2 hours before your training session.

**DURING EXERCISE INSTRUCTIONS:**

1. Ensure you record the time of day when you start your session.
2. Remember that your running speed and duration will change throughout the training programme. Check each training week's page for the speed/intensity required.
3. Please only consume water during your exercise runs.

**POST-EXERCISE INSTRUCTIONS:**

1. Record the duration of your session in minutes.
2. Remember to consume your first sports drink **immediately** after exercise (i.e. within 5 minutes).
3. Your second sports drink should be consumed **precisely an hour after** your first drink.
4. Please do not consume any calories other than the drinks provided for the 2 hour period after exercise.

**Baseline Testing Week**

Please tick or fill in boxes where appropriate

<b>Session</b>	<b>1</b>	<b>2</b>	<b>-</b>	<b>-</b>
<b>Date</b>	/ /	/ /		
<b>Before Exercise</b>				
<b>Eaten 2-h before exercise</b>				
<b>Consumed no other calories between this meal and exercise</b>				
<b>Exercise</b>				
<b>Time started</b>	:	:		
<b>Duration/Time finished</b>	mins	mins		
<b>After exercise</b>				
<b>Consumed sports drink immediately after exercise</b>				
<b>Consumed sports drink 1 hour after exercise</b>				
<b>No Calories consumed 2 hours after exercise</b>				

Speed @ 70%  $\dot{V}O_{2\max}$  .....

Duration of session: 30 minutes

Notes:

**First Training Week**

Please tick or fill in boxes where appropriate

Session	1	2	3	4
Date	/ /	/ /	/ /	/ /
<b>Before Exercise</b>				
Eaten 2-h before exercise				
Consumed no other calories between this meal and exercise				
<b>Exercise</b>				
Time started	:	:		
Duration/Time finished	mins	mins		
<b>After exercise</b>				
Consumed sports drink immediately after exercise				
Consumed sports drink 1 hour after exercise				
No Calories consumed 2 hours after exercise				

Speed @ 70%  $\dot{V}O_{2\max}$  .....

Duration of session: 40 minutes

Notes:

### **Estimation of protein oxidation rates**

Nutrient provision in **Chapter 5** involved the co-ingestion of protein with carbohydrate during a 4 h post-exercise recovery period at an ingestion rate of 0.4 g protein·kg BM<sup>-1</sup>·h<sup>-1</sup>. Thus, RER values during recovery were corrected using urinary urea excretion to estimate protein oxidation (Jequier *et al.*, 1987). Following Run-1, urine was collected in a vessel containing a preservative (5 ml of 10 % thymol-isopropanol) and preceded throughout the 4 recovery period. Once total urine output during this period was recorded, a mixed 1 ml sample was taken and stored at -80 °C. The urea concentration (in mmol·l<sup>-1</sup>) of this sample was then used as an estimate of total urine nitrogen excretions. Plasma urea concentrations were subsequently used to correct urinary urea excretion for whole-body determination of urea pool during recovery (Livesey and Elia, 1988). Non-protein respiratory exchange ratio (NPRER) was then calculated to reflect protein oxidation rates during the 4 h recovery phase. Below is a step-by-step example to correct RER values using urinary urea excretion to estimate non-protein RER for one participant (**65.8 kg; urine volume during recovery= 570 ml; urine urea concentration in recovery= 178.4 mmol·l<sup>-1</sup>**).

- 1) Obtain urea concentration in g·ml<sup>-1</sup>: divide 178.4 mmol·l<sup>-1</sup> by 35700 (urea nitrogen conversion factor from mmol·l<sup>-1</sup> to mg·dl<sup>-1</sup>= 0.357). The yield is 0.005 g·ml<sup>-1</sup>.
- 2) Estimate urinary nitrogen in g·ml<sup>-1</sup> from urea by multiplying by 0.47 (as nitrogen only represents 47% of urea). The yield is 0.0023 g·ml<sup>-1</sup>.
- 3) Calculate total nitrogen loss during recovery in g by multiplying by total urine volume produced during recovery (i.e. 570 ml). This yields 1.34 g.
- 4) Divide total nitrogen loss during recovery (1.34 g) by total recovery time in min (i.e. 240 min) to obtain nitrogen loss per minute. The yield is 0.006 g·min<sup>-1</sup>.
- 5) Total nitrogen loss per minute is then multiplied by a factor 6.25 (as nitrogen only comprises ≈16% of protein) to obtain protein oxidation rates. This yields 0.03 urine g·min<sup>-1</sup>.



**The estimate of protein oxidation rate in urine can then be corrected for bodily urea pool by using plasma urea data from the participant:**

- 1) Obtain the change in plasma urea in recovery by subtracting post- from pre-recovery plasma urea values (i.e.  $7.51 - 4.92 \text{ mmol}\cdot\text{l}^{-1}$ ). The yield is  $2.59 \text{ mmol}\cdot\text{l}^{-1}$ .
- 2) Convert  $\text{mmol}\cdot\text{l}^{-1}$  to  $\text{g}\cdot\text{l}^{-1}$  (multiply by the molecular weight of urea= 60). This yields  $155.4 \text{ g}\cdot\text{l}^{-1}$ .
- 3) Multiply by 0.47 ( $\approx 47\%$  of urea is nitrogen). The yield is  $73.04 \text{ g}\cdot\text{l}^{-1}$ .
- 4) Obtain whole body nitrogen estimation in g by multiplying the above value by body mass  $\times 0.57$  (i.e.  $\approx 57\%$  of total body mass is body water). This yields  $2739.36 \text{ g}$ .
- 5) The value is divided by  $1000 \times$  recovery time (240 min) to obtain plasma change in nitrogen  $\text{g}\cdot\text{min}^{-1}$ . The yield is  $0.114 \text{ g}\cdot\text{min}^{-1}$ .
- 6) Multiply by 6.25 to yield protein oxidation rate (nitrogen comprises  $\approx 16\%$  of protein). This yields  $0.07 \text{ plasma g}\cdot\text{min}^{-1}$ .
- 7) Protein oxidation rate obtained from urine (i.e.  $0.03 \text{ urine g}\cdot\text{min}^{-1}$ ) can be corrected for change in bodily urea pool through the addition of the estimate of plasma protein oxidation rate (i.e.  $0.07 \text{ plasma g}\cdot\text{min}^{-1}$ ). Therefore, the corrected protein oxidation rate would be  $0.11 \text{ g}\cdot\text{min}^{-1}$ .
- 8) Protein (p)  $\dot{V}\text{O}_2$  and  $\dot{V}\text{CO}_2$  are calculated (1 g of protein requires the consumption of 0.966 l of  $\text{O}_2$  and produces 0.782 l of  $\text{CO}_2$ ). Therefore, the corrected protein oxidation rate ( $0.11 \text{ g}\cdot\text{min}^{-1}$ ) is subtracted from these values to obtain p  $\dot{V}\text{O}_2$  and p  $\dot{V}\text{CO}_2$  (e.g.  $0.11 - 0.966 = 0.10 \text{ l}\cdot\text{min}^{-1}$ ). The yield is  $0.10$  and  $0.08 \text{ l}\cdot\text{min}^{-1}$  for p  $\dot{V}\text{O}_2$  and p  $\dot{V}\text{CO}_2$ , respectively.
- 9) This correction factor is subtracted from the values obtained from indirect calorimetry (e.g.  $\dot{V}\text{O}_2$  and  $\dot{V}\text{CO}_2$  at 4<sup>th</sup> hour of recovery=  $0.4$  and  $0.4 \text{ l}\cdot\text{min}^{-1}$ , respectively) to obtain non-protein  $\dot{V}\text{O}_2$  and  $\dot{V}\text{CO}_2$  to establish corrected substrate oxidation rates. Thus, following correction  $\dot{V}\text{O}_2$  and  $\dot{V}\text{O}_2$  values would be  $0.3 \text{ l}\cdot\text{min}^{-1}$  and  $0.3 \text{ l}\cdot\text{min}^{-1}$ , respectively.
- 10) Accordingly, NPRER was calculated based on the non-protein substrate oxidation rates to yield NPRER ( $0.3/0.3=1$ ).

## **Acid Hydrolysis of Muscle Sample**

### **Method:**

- 1) Make up the following reagent mixtures:
- 2) HCl 1mol/L
- 3) NaOH 6mol/L
- 4) For each muscle sample add 0.1ml of HCl per mg of muscle powder to the precipitated muscle pellet.
- 5) Add 0.1ml of HCl to the 20 $\mu$ l of undiluted neutralised extract.
- 6) Mix both samples gently and incubate for two hours in tightly screwed Eppendorf tubes in a boiling water bath.
- 7) Centrifuge tubes for 1 min and leave at room temperature to cool.
- 8) Add 15 $\mu$ l of NaOH to neutralise the acid hydrolysed extract.

Acid insoluble and soluble glycogen to be assayed spectrophotometrically using a method to determine glucose concentration (see attached protocol)

## Measurement of Creatine

### Principle:

Pyruvate + NADH  $\xrightarrow{\text{LDH}}$  Lactate + NAD

ADP + PEP  $\xrightarrow{\text{PK}}$  ATP + Pyruvate

Cr + ATP  $\xrightarrow{\text{CPK}}$  PCr + ADP

### Method:

1) Make up the following reagent mixture using these quantities per well:

D4	(Glycine buffer)	75 $\mu$ l
D3	(KCl 15g/100ml)	3.75 $\mu$ l
ATP	(15.4mg/ml)	15 $\mu$ l
PEP	(11.6mg/ml)	11.25 $\mu$ l
NADH	(9mg/ml)	3.75 $\mu$ l
LDH		0.375 $\mu$ l
PK		0.375 $\mu$ l
Dist H <sub>2</sub> O		115.5 $\mu$ l
		<b>225<math>\mu</math>l</b>

Vortex and keep on ice

2) Make standard (anhydrous, Sigma C-0780, MW 131.1).

3) Prepare the enzyme:

CPK (Sigma C-3755). Dissolve 15mg/ml in 0.5% NaHCO<sub>3</sub> + 0.05% BSA (D5).

Use 5 $\mu$ l per well.

Vortex and keep on ice.

4) Defrost samples quickly in hot water, vortex and spin down (14000rpm, 3min)

5) Set the plate reader to read samples at 340nm.

6) Pipette 225 $\mu$ l of the reagent mix into each well.

7) Add 15 $\mu$ l of water/standard/sample into each well.

8) Read absorbance (A1).

9) Add 5 $\mu$ l of the CPK. Incubate for 30min.

10) Read absorbance (A2).

**Calculations:** (units = mmol/kg dm weight)

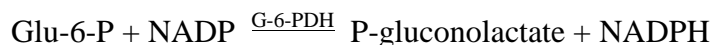
$$\text{Conc.} = \frac{((\text{final vol} * (\text{Ab}_n - \text{blank}_n)) - (\text{vol before enzyme} * (\text{Ab}_{n-1} - \text{blank}_{n-1}))) * \text{ext factor} * 1.25 * \text{dil factor}}{6.22 * \text{volume of sample}}$$

Notes:

- Remember that volumes increase as each subsequent enzyme is added.
- Ext factor is extraction factor
- Dil factor is dilution factor
- 6.22 is extinction factor

## Measurement of ATP, PCr and G-6-P

### Principle:



### Method:

1) Make up the following reagent mixture using these quantities per well:

D1	(TEA pH 7.5-7.6 buffer)	22.2µl
DTT	(7.8mg/ml)	4.4µl
NADP	(20.9mg/ml)	8.9µl
ADP (	(5.1mg/ml)	0.9µl
Glucose	(22.5mg/ml)	8.9µl
Dist H <sub>2</sub> O		154.7µl
		<hr/> 200µl

Vortex and keep on ice

2) Make 1 mM standards for G6P, ATP and PCR. Combine standards into one well.

3) Dilute the following enzymes:

- a. Glucose-6-Phosphate-Dehydrogenase (Sigma G-5885).  
Dilute 1 part enzyme to 1 part Dist H<sub>2</sub>O. Use 3µl per well.
- b. Hexokinase (HK) (Roche 1500U/ml). Dilute 1 part enzyme to 1 part Dist H<sub>2</sub>O. Use 5µl per well.
- c. Creatine phosphokinase (CPK) (Sigma C-3755). Dissolve 15mg/ml in 0.5% NaHCO<sub>3</sub> + 0.05% BSA (D5). Dilute 2 parts enzyme to 1 part Dist H<sub>2</sub>O. Use 3µl per well.

Vortex and keep on ice.

- 4) Defrost samples quickly in hot water, vortex and spin down (14000rpm, 3min)
- 5) Set the plate reader to read samples at 340nm.
- 6) Pipette 200µl of the reagent mix into each well.
- 7) Add 20µl of water (blank)/sample. Add 21µl (3x7 µl of each standard) to a single well.
- 8) Read absorbance (A1).
- 9) Add 3µl of the G6PDH. Incubate for 10-15 min.
- 10) Read absorbance (A2).
- 11) Add 5µl of the HK. Incubate for 15-20 min.

12) Read absorbance (A3).

13) Add 3µl of the CPK. Incubate for 30-35 min.

14) Read absorbance (A4).

**Calculations:** (units = mmol/kg dm weight)

$$\text{Conc.} = \frac{((\text{final vol} * (\text{Ab}_n - \text{blank}_n)) - (\text{vol before enzyme} * (\text{Ab}_{n-1} - \text{blank}_{n-1}))) * \text{ext factor} * 1.25 * \text{dil factor}}{6.22 * \text{volume of sample}}$$

Notes:

- Remember that volumes increase as each subsequent enzyme is added.
- Ext factor is extraction factor
- Dil factor is dilution factor
- 6.22 is extinction factor

## **Measurement of Glucose (Insoluble and soluble glycogen)**

### **Method:**

1) Make up the following reagent mixture using these quantities per well:

G1	Triethanolamine (TEA)	7.00g/100ml	64µl
	KOH	0.80g/100ml	
	Mg (AC) <sub>2</sub> .4H <sub>2</sub> O	2.40g/100ml	
	EDTA Na <sub>2</sub> .2H <sub>2</sub> O	0.14g/100ml	
	pH=8.2 (KOH)		
ATP		27.72mg/ml	4µl
DTT		9.36mg/ml	4µl
NAD		19.92mg/ml	8µl
H <sub>2</sub> O			120µl
			□ 200 µl

Vortex and keep on ice

2) Make a glucose standard (1.5mmol/l): 2.70 mg/10 ml of water)

3) Dilute the following enzymes:

G-6-PDH	Sigma G-5885, 200 units/mg	Reconstitute with 200µl H <sub>2</sub> O
HK	Sigma H-4502, 256 units/mg	Reconstitute with 256µl H <sub>2</sub> O
Mix equal parts of G-6-PDH and HK (ie 50µl + 50µl)		

Vortex and keep on ice.

4) Defrost samples quickly in hot water, vortex and spin down (14000rpm, 3min)

5) Set the plate reader to read samples at 340nm

6) Pipette 200µl of the reagent mix into each well.

7) Add 20µl of water/standard/sample.

8) Read absorbance (A1).

9) Add 2µl of the mixed enzyme and incubate for 15-20 min.

10) Read final absorbance (A2).

**Calculations:** (units = mmol glucosyl units/kg dm weight)

$$\text{Conc.} = \frac{[(A2 \times V2) - (A1 \times V1)] - [(AB2 \times V2) - (AB1 \times V1)] \times \text{Extraction Factor}}{\text{Standard Volume} \times 6.22}$$

[6.22 is Extinction factor]

## **RNA Extraction from Muscle Tissue**

### **Preparation:**

- 1) Clean processing area with 100% ethanol
- 2) Label up (top and sides) all relevant tubes to be used in extraction protocol
  - a. 100% Ethanol tubes for cleaning homogeniser (1 tube per 4 samples)
  - b. DEPC water tubes for cleaning homogeniser ( 1 tube per 4 samples)
  - c. Large conical tubes for homogenising each sample
  - d. Large conical tube for chloroform, ethanol and DEPC water
  - e. Small conical tube for centrifuging and pipetting out aqueous phase (AP)
  - f. Small conical tube for adding ethanol to AP
  - g. Eppendorf's for each sample (1 for each elution)
- 3) Get appropriate pipette tips ready (ideally use filtered tips):
  - a. P1000                200 – 1000 µl
  - b. P200                20 – 200 µl
  - c. P10                0.5 – 10 µl
- 4) Check pipette accuracy by weighing drawn quantity
- 5) Get a scalpel for cutting samples down where needed
- 6) Have blue towel, ethanol and tweezers handy for cleaning homogeniser
- 7) Get centrifuges set up with appropriate rotor and speed:
  - a. Large centrifuge needs medium and large conical base with 2575 g.min, - 4°C.
  - b. Small centrifuge needs 13,000 rpm
- 8) Place larger glass beaker in both work spaces for discarding tips and tubes
- 9) Prepare buffer for columns by adding quantities of ethanol stated on each
- 10) Get a box of crushed ice and a container of liquid nitrogen.
- 11) In fume hood, pipette 2 ml TRIzol in to each conical tube (same tip)

### **Homogenisation:**

- 1) Place cap of large conical tube on scales and zero
- 2) Run fingers around biopsy container and flick to detach sample.
- 3) Tip contents on to cap using tweezers if needed, looking for 20-30 milligrams.
- 4) Return to liquid nitrogen, cut sample and re-weigh as needed.
- 5) Drop sample from nitrogen in to TRIzol using tweezers (smallest piece first).
- 6) Homogenise sample on crushed ice until fully disrupted (i.e. no visible chunks left)
  - a. Trap sample in barrel, starting at low speed before increasing
  - b. Run round and round in fluid, up and down in fluid, spin off excess, tweeze trapped chunks back in to TRIzol and repeat.
- 7) Fridge sample immediately once homogenised
- 8) Note weight alongside sample ID
- 9) Return remaining sample to biopsy tube from liquid nitrogen and freeze
- 10) Clean homogeniser of residue and connective tissue:
  - a. Spin through ethanol then RNAase free water
  - b. Tweeze out visible tissue



- c. Wipe contact elements with ethanol paying close attention to the joints
  - d. Spin through ethanol and RNAase free water
  - e. Check and repeat if needed
- 11) Repeat for next sample.

### Separation:

- 1) Remove samples from fridge, spin in centrifuge for 1 min.
- 2) Pipette samples from wide conical tubes to narrower conical tubes.
- 3) Pipette in 400 µl of chloroform (does not need to be exact)
  - a. Needs to be in ratio to TRIzol volume (700 µl TRIzol : 140 µl Chloroform)
- 4) **Screw lid on tightly** and shake each sample vigorously for 15 seconds
- 5) Return samples to rack and leave at room temperature for 2-3 minutes to separate
- 6) Check lids and centrifuge for 15 minutes
  - a. Gives organic phase (OP) with proteins in bottom and aqueous phase (AP) with RNA on top
  - b. Label up columns while you wait
- 7) Use P1000 tip to transfer AP to next set of small conical tubes.
  - a. Avoid taking up OP
  - b. Can spin to separate again if they become mixed
- 8) Place APs on ice and repeat for next sample
- 9) Freeze remaining OPs (could be used for western blot).
- 10) **Pipette 1500 µl (2 x 750) of 100% ethanol in to AP**
  - a. **Ethanol volume should be 1.5x the volume of AP**
- 11) **Pipette up and down to thoroughly mix (can place on weak vortex to enhance)**
  - a. **Hold against dark background; solution should go cloudy to show it is precipitating**
  - b. **Forgetting to add ethanol or mix appropriately will cause RNA to be lost**

### Extraction:

- 1) Load AP on to columns using Pasteur pipettes (stay below rim).
  - a. Draw all AP in to pipette first
  - b. Leave pipette in corresponding conical tube for repeats
- 2) Place in centrifuge and spin at  $\approx 13000$  for 30-60 seconds
- 3) Remove from centrifuge and discard liquid from column holder
  - a. Remove column from holder, discard fluid from holder in to a beaker, tap on holder hard on blue towel to drain, place column back in holder.
- 4) Pipette next batch of AP and repeat until all AP is filtered.
  - a. **Make sure you do not get columns mixed up.**
- 5) Add 400 µl of RWT buffer to each column, spin and empty holders as before
  - a. Pipetting needs to be precise
- 6) Add another 400 µl of RWT buffer to each column, spin and empty holders as before
  - a. Pipetting needs to be precise
- 7) Add 500 µl of RPE buffer to each column, spin and empty holders as before
  - a. Pipetting needs to be precise

- 8) Add another 500 µl of RPE buffer to each column, spin and empty holders as before
  - a. Pipetting needs to be precise
- 9) Spin at 13,000 with nothing further added for 2 minutes
- 10) Discard fluid and use blue towel to remove all on outside of column
  - a. Repeat 9-10 until fluid stops appearing
  - b. Trying to remove all ethanol as it will contaminate RNA
- 11) Open column top and place in Elution 1 RNA Eppendorf for 10-15 minutes for evaporation
  - a. Remaining drops in the column itself can be pipetted out.
  - b. Place in culture hood with vacuum on.
  - c. Can set up Spectrophotometer while waiting.

## Quantitation:

- 1) Add 25 µl of RNAase free water from extraction kit to centre of column membrane
  - a. Either just above or touch and withdraw
- 2) Spin at 13,000 for 2 minutes with Eppendorf lids open and facing down
  - a. RNA is contained in fluid in Eppendorf after spinning
  - b. Can use a pipette to check volume
- 3) Launch 'Spectrostar Nano'
  - a. > User > Run > LVis Tab (use Microplate for ELISA)
  - b. Insert empty plate (A1 in top left)
  - c. > Cleanliness Check > Measure
  - d. Want values of 0.045 or less, if not clean with DEPC water and recheck
  - e. Remove plate and lift lid on RHS of plate
  - f. Pipette 2 µl of sample on to dot (P10 tips)
  - g. Insert plate > RNA
  - h. Top middle icon, 'MARS' > Double-click target sample > Select 260/280 and 260/230 ratios on LHS > Table View > Export to Excel
  - i. Organise in to table 'RNA-quantitation\_table-blank'

Nucleotides, RNA, ssDNA, and dsDNA all will absorb at 260 nm and contribute to the total absorbance.

The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of DNA and RNA. A ratio of  $\approx 1.8$  is generally accepted as "pure" for DNA; a ratio of  $\approx 2.0$  is generally accepted as "pure" for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

260/230 ratio is used as a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants which absorb at 230 nm. EDTA, carbohydrates and phenol all have absorbance near 230 nm. The TRIzol reagent is a phenolic solution which absorbs in the UV both at 230 nm and  $\approx 270$  nm.

## **cDNA Conversion from RNA**

### *SuperScript III First-Strand Synthesis System for RT-PCR*

#### **Preparation:**

- 1) Thoroughly wipe down benching and equipment with ethanol.
- 2) Label up (top and sides) all tubes to be used in protocol:
  - a. 1 x DEPC water tube for dilution.
  - b. Eppendorfs for all samples to be converted (1 per sample).
- 3) Get appropriate pipette tips ready (ideally use filtered tips):
  - a. P200                      20 – 200  $\mu$ l
  - b. P10                        0.5 – 10  $\mu$ l
  - c. 125  $\mu$ l tips for electronic pipette.
- 4) Check pipette accuracy by weighing drawn quantity.
- 5) Place clean glass beaker in workspace for discarding tips.
- 6) Set up centrifuge (small), vortex (set to 16) and block heater/incubator.
- 7) Get two boxes of crushed ice.

#### **Dilution:**

- 1) Aim is to create an 8.0  $\mu$ l solution containing 50 ng of RNA per  $\mu$ l:
  - a. Divide 400 ng by the RNA concentration calculated in the RNA quantitation.
  - b. Gives volume ( $\mu$ l) of RNA elution required to give 400 ng of RNA.
  - c. Subtract this value from 8.0  $\mu$ l to get volume of DEPC water needed to dilute.
- 2) Pipette calculated volume of DEPC water in to corresponding Eppendorf:
  - a. Will be different for each sample.
  - b. Complete for all samples before proceeding.
  - c. Tick off this step on the protocol so you know it has been added.
- 3) Remove RNA elutions from the freezer, place in order and defrost at room temperature.
- 4) Once defrosted mix on a weak vortex and briefly centrifuge to collect before placing on ice:
  - a. Keep all Eppendorfs on ice from here onwards
- 5) Pipette calculated volume of RNA elution in to corresponding Eppendorf:
  - a. Will be different for each sample.
  - b. Complete for all samples before proceeding.
  - c. Tick off this step on the protocol so you know it has been added.
  - d. Return what remains of elutions to the freezer.
- 6) Mix DEPC-RNA on a weak vortex, collect by brief centrifugation and return to ice.

#### **cDNA Synthesis**

- 1) Remove 'Superscript III' kit from freezer and place on ice.
- 2) Remove 'Random Hexamers' and '10mM dNTP Mix' to defrost at room temperature:
  - a. Can use weak vortex and holding to accelerate defrost.
  - b. Check both are free from ice by visual inspection before continuing.
- 3) Label up a new Eppendorf with 'Hex + DNTP'.
- 4) Add 1  $\mu$ l of each component to the 'Hex + DNTP' for each sample to be converted:
  - a. i.e. for 50 samples, 50  $\mu$ l of 'Random Hexamers' and 50  $\mu$ l of '10mM dNTP Mix' are needed.
- 5) Mix in Eppendorf using pipette before vortexing and centrifuging to collect.

- 6) Set block heater/incubator to 65°C.
  - a. Hold 'Set' and adjust temperature as needed.
- 7) Use electronic pipette to add 2 µl of the 'Hex + dNTP' solution to each Eppendorf:
  - a. Place on the side of the Eppendorf so reaction doesn't start.
  - b. Can use same tip provided you don't touch the sample.
  - c. Remember to keep the Eppendorfs on ice as you do this.
- 8) Briefly centrifuge each Eppendorf to combine the mix with the diluted RNA before vortexing weakly to mix and centrifuging again to collect.
- 9) Place Eppendorfs in the block heater to incubate at 65°C for 5 minutes.
- 10) Remove blocks containing Eppendorfs from the block heater and place on ice for at least 1 minute:
  - a. Make sure water does not submerge Eppendorfs as the ice melts.
- 11) While the blocks are cooling remove the components listed in table 1 from the 'Superscript III' kit to defrost at room temperature:
  - a. Can use weak vortex and holding to accelerate defrost.
  - b. Check both are free from ice by visual inspection before continuing.

Table 1: cDNA Synthesis Mix components and quantities.

Component	1 Sample	10 Samples
10X RT Buffer	2 µl	20 µl
25 mM MgCl <sub>2</sub>	4 µl	40 µl
0.1 M DTT	2 µl	20 µl
RNaseOUT (40 U/µl)	1 µl	10 µl
SuperScript III RT (200 U/µl)	1 µl	10 µl

- 1) Label up a new Eppendorf with 'Synth. Mix'.
- 2) Mix each of the listed components by vortexing and collect by brief centrifugation.
- 3) Pipette an appropriate quantity of each component in to the 'Synth. Mix' Eppendorf based on the number of samples you are converting:
  - a. Add the components in the order they are listed in.
  - b. Mix with the pipette at each end to ensure homogeneity.
- 4) Once all the components have been added, mix on a weak vortex and centrifuge to combine:
  - a. While centrifuging dry the incubator blocks and return them to the incubator.
  - b. Set incubator temperature to 25°C.
- 5) Use an electronic pipette to add 10 µl of the 'Synth. Mix' to each Eppendorf:
  - a. As before, pipette it on to the side rather than directly in to the sample so the reaction doesn't start until it is centrifuged.
- 6) Once the cDNA Synthesis Mix has been added to all Eppendorfs centrifuge them to combine, place on a weak vortex to mix and centrifuge again to collect.

**Incubation:**

- 1) Ensure incubator temperature has stabilised at 25°C:
  - a. We used room temperature which was set to 25°C
- 2) Place Eppendorfs in to the incubator at 25°C for 10 minutes.
- 3) After 10 minutes increase the incubator temperature to 50°C.
- 4) Incubate for a further 50 minutes at 50°C.

- 5) After 50 minutes increase the incubator temperature to 85°C.
- 6) Incubate for 5 minutes at 85°C to terminate the reaction.
- 7) Remove blocks containing Eppendorfs from the block heater and place on ice:
  - a. Make sure water does not submerge Eppendorfs as the ice melts.
- 8) Once the Eppendorfs are cooled collect the samples by brief centrifugation and freeze.

## **Percentage plasma volume change and the estimation of plasma albumin content**

In **Chapter 7**, plasma albumin content was determined from plasma albumin concentration (in  $\text{mmol}\cdot\text{l}^{-1}$ ) and the percentage change in plasma volume. Plasma albumin concentrations were determined using a spectrophotometric analyser (RX Daytona, Randox Laboratories Ltd., Crumlin, UK). Blood haemoglobin and haematocrit (described in further detail in general methods section) concentrations were used to determine the percentage change in plasma volume in procedures described previously (Dill and Costill, 1974).

### **A) Determination of percentage plasma volume change based on haemoglobin and haematocrit:**

As described by Dill and Costill (1974), percentage change in blood volume (BV), red cell volume (CV) and plasma volume (PV) can be calculated from values from haemoglobin (Hb) and haematocrit (Hct) before (pre-exercise in **Chapters 4 and 5** and at rested baseline in **Chapter 7**) and after (each time point during running/recovery in **Chapters 4 and 5** and at rested follow-up in **Chapter 7**) a given time point. Accordingly, the subscripts B and A refer to before and after, respectively, and  $BV_B$  was taken as 100.

$$1) \quad BV_A = BV_B \times (Hb_B/Hb_A)$$

$$2) \quad CV_A = (BV_A \times Hct_A) / 100$$

$$3) \quad PV_A = BV_A - CV_A$$

$$\text{Percentage change in plasma volume (PV \%)} = (PV_A - PV_B) / PV_B \times 100$$

Where  $PV_B$  was taken as  $100 - Hct_B$

**B) Estimations of plasma albumin content**

Participants were assumed to have a plasma volume (in L) equivalent to 5 % of body mass (in kg) at baseline and plasma volume at follow-up was determined using baseline plasma volume and the relative change in plasma volume. Thus (based on an example of a 70 kg participant with a percentage plasma volume change of 4%; baseline plasma albumin concentration of  $41.3 \text{ g}\cdot\text{L}^{-1}$  and post-intervention albumin concentration of  $42.6 \text{ g}\cdot\text{L}^{-1}$ ):

- 1) Multiply body mass by 0.05 (plasma volume in L in humans at rest is  $\approx 5\%$ ) to obtain baseline plasma volume. This yields 3.5 L.
- 2) Then, multiply baseline plasma volume by the change in plasma volume ( $3.5 \times 4$ ) /100. This yields 0.14 L.
- 3) Therefore, plasma volume change from baseline in L is ( $3.5 \text{ L} + 0.14 \text{ L} = 3.64 \text{ L}$ ).
- 4) Change baseline plasma albumin concentration (in  $\text{g}\cdot\text{L}^{-1}$ ) to plasma albumin content ( $\text{g}\cdot\text{kg}^{-1}$ ) by multiplying plasma albumin concentration by baseline plasma volume in L and divide by body mass (i.e.  $(41.3 \text{ g}\cdot\text{L}^{-1} \times 3.5 \text{ L})/70 \text{ kg}$ ). This yields an albumin content of  $2.06 \text{ g}\cdot\text{kg}^{-1}$ .
- 5) Perform the same calculations as the previous step to post-intervention measurements to obtain plasma albumin content at follow-up (i.e.  $(42.6 \text{ g}\cdot\text{L}^{-1} \times 3.64 \text{ L})/70 \text{ kg}$ ). Plasma albumin content at follow-up would therefore be  $2.21 \text{ g}\cdot\text{kg}^{-1}$ .

### Calculation of Confidence Intervals

In the present thesis, where appropriate, the error bars on figures are confidence intervals (CI) that have been corrected to remove between subject variance (Loftus and Masson, 1994b). Presented below is an example of the CI calculation used for Run-1 times to exhaustion. Specifically, the CI about the mean run times to exhaustion in **Chapter 4** (L-CHO *versus* H-CHO treatments) were calculated.

1) A general linear model was performed on the data using SPSS:

Tests of Within-Subjects Effects

Measure: MEASURE\_1

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Run-1	Sphericity Assumed	451.250	1	451.250	2.816	.128
	Greenhouse-Geisser	451.250	1.000	451.250	2.816	.128
	Huynh-Feldt	451.250	1.000	451.250	2.816	.128
	Lower-bound	451.250	1.000	451.250	2.816	.128
Error(factor1)	Sphericity Assumed	1442.250	9	160.250		
	Greenhouse-Geisser	1442.250	9.000	160.250		
	Huynh-Feldt	1442.250	9.000	160.250		
	Lower-bound	1442.250	9.000	160.250		

2) The Mean Square value for error and the corresponding degrees of freedom (df) were noted: 160.250 and 9, respectively.

3) A t-distribution was consulted to determine a t value for df = 9:  $t_{(9)} = 2.262$ .

4) A normalised CI was then calculated using the formula:

$$CI = M_j \pm \sqrt{\frac{MS_w}{n}} [\text{criterion } t(27)],$$

Where: CI = confidence interval

$M_j$  = mean of condition (i.e. raw data)



$MS_{sxc}$  = mean squared error

n = number of subjects in each trial

criterion t = t-distribution value

So for the given example this formula was:

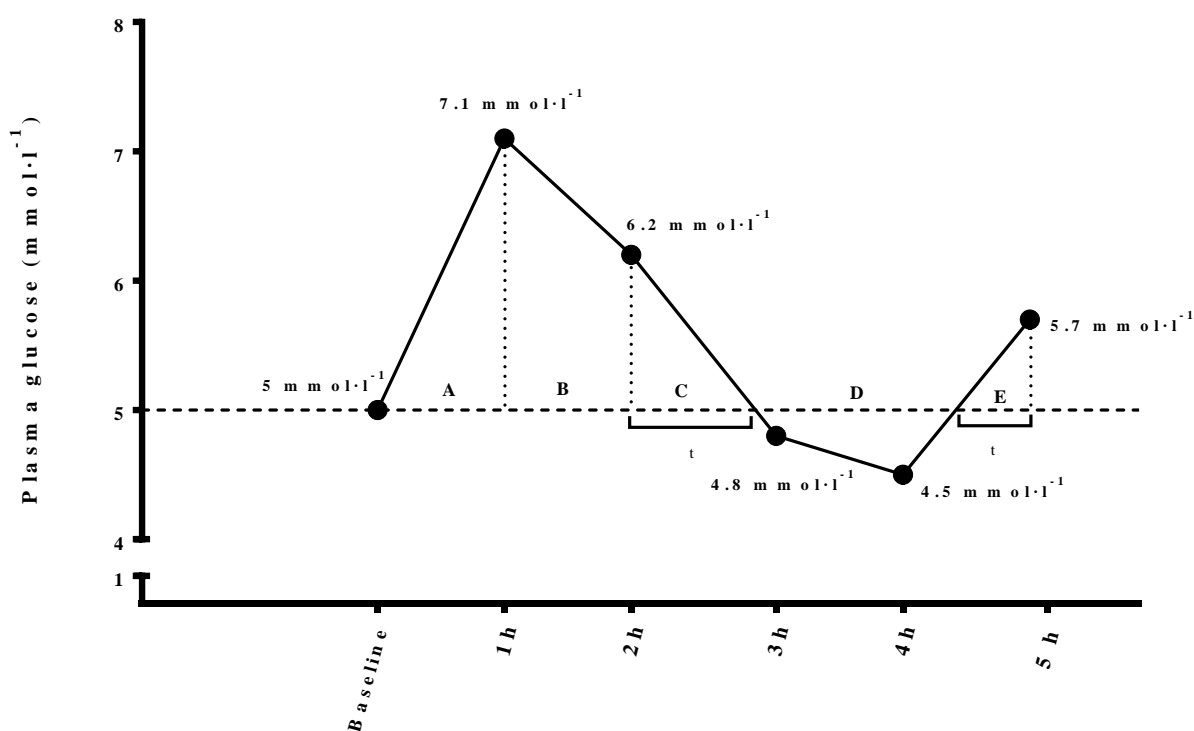
$$= (\sqrt{160.250/10}) \times 2.262$$

$$= 9.1$$

5) Thus, the overall CI that can be plotted about the mean in this example is **9.1** min for both the L-CHO and H-CHO trials.

### Calculation of incremental area under the curve

In **Chapters 4** and **5**, the incremental area under the concentration curve (iAUC) for plasma glucose and serum insulin during the 4 h recovery period were calculated by incorporating the method recommended by Wolever (Wolever, 2004). A step-by-step description of calculating iAUC is provided in the example below.



1) **Triangle A** = (start concentration + end concentration) × ½ time (min) – baseline area

Thus,  $(5 + 7.1) \times 30 - (5 \times 60)$  and the result would be **63 mmol·l<sup>-1</sup>**

2) **Trapezoid B** =  $(7.1 + 6.2) \times 30 - (5 \times 60)$  and the result would therefore be **99 mmol·l<sup>-1</sup>**

3) **Triangle C** = (start concentration – baseline concentration) × t / 2

where  $t = (\text{start concentration} - \text{baseline concentration}) / (\text{start concentration} - \text{end concentration}) \times \text{time (min)}$ .

Therefore,  $t = (6.2 - 5) / (6.2 - 4.8) \times 60$  and the result is 51.4 min

So, Triangle C =  $(6.2 - 5) \times 51.4/2$  and the result would be **30.8 mmol·l<sup>-1</sup>**

4) **Period D** = both the start and end concentrations for this period are below baseline so  
iAUC = **0 mmol·l<sup>-1</sup>**

5) **Triangle E** = (end concentration – baseline concentration) × t / 2

where t = (end concentration – baseline concentration) / (end concentration + start concentration) × time (min).

Therefore, t =  $(5.7 - 5) / (5.7 + 4.5) \times 60$  and the result is 35 min

So, Triangle E =  $(5.7 - 5) \times 35/2$  and the result would be **12.2 mmol·l<sup>-1</sup>**

6) The total iAUC is the sum of all periods in the example (A+B+C+D+E)

Thus,  $63+99+30.8+0+12.2=$  **205 mmol·300 min·l<sup>-1</sup>**